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**TITLE OF THE INVENTION**

Recombinant *P. falciparum* Merozoite Protein-1<sub>42</sub> Vaccine

This application claims the benefit of priority  
5 under 35 U.S.C. §119(e) from U.S. application serial  
no. 60/264,535 filed on January 26, 2001, still  
pending, and U.S. provisional application filed on  
October 26, 2001, still pending.

**INTRODUCTION**

10 *Plasmodium falciparum* is the leading cause of  
malaria morbidity and mortality. The World Health  
Organization estimates that approximately 200 million  
cases of malaria are reported yearly, with 3 million  
15 deaths (World Health Organization, 1997, Wkly.  
Epidemiol. Rec. 72:269-276). Although, in the past,  
efforts have been made to develop effective controls  
against the mosquito vector using aggressive  
applications of pesticides, these efforts ultimately  
20 led to the development of pesticide resistance.  
Similarly, efforts at treatment of the disease through  
anti-parasitic drugs led to parasite drug-resistance.  
As the anti-vector and anti-parasite approaches  
failed, efforts became focused on malaria vaccine  
25 development as an effective and inexpensive  
alternative approach.

However, the complex parasitic life cycle has  
further confounded the efforts to develop efficacious  
vaccines for malaria. The parasite's life cycle is  
30 divided between the mosquito-insect host and the human  
host. While in the human host, it passes through  
several developmental stages in different organellar  
environments, i.e. the liver stage, the red blood  
stage. Although conceptually simple, in reality the

problems that must be considered when designing subunit vaccines for malaria are great. Antigen diversity is a characteristic that must be taken into account and includes a high degree of developmental stage specificity, antigenic variation and antigen polymorphism. Vaccine candidates have been identified from each of the parasite's developmental stages. The major merozoite surface protein-1, MSP-1, is among the leading erythrocytic stage vaccine candidates (Diggs, et al, 1993, Parasitol. Today 9: 300-302). The objective of erythrocytic stage vaccines is to diminish the level of parasitemia in the bloodstream and thus reduce the severity of disease.

Although the MSP-1 molecule has been studied extensively, its function is not fully understood. There is evidence that MSP-1 binds to erythrocytes and may have a role in erythrocyte invasion (Perkins and Rocco, 1988, J. Immunol. 141, 3190-3196; Holder, A. A., 1994, Parasitology 108 (Suppl.) S5-18).

MSP-1 is secreted as a membrane-anchored (Haldar et al., 1985, J. Biol. Chem. 260, 4969-4974) 195 kDa precursor that is proteolytically processed to products with nominal molecular masses of 83, 28-30, 38-45, and 42 kDa during merozoite development (Holder and Freeman, 1984, Phils Trans R. Soc. Lond B. Bio. Sci. 307, 171-177; Lyon et al., 1987, J. Immunol, 138, 895-901; Holder et al., 1987, Parasitology 94, 199-208). These protein fragments form a non-covalent complex on the surface of merozoites (McBride and Heidrich, 1987, Parasitology 23, 71-84; Lyon, et al., 1987, supra) that remain attached to the merozoite surface through the C-terminal 42 kDa fragment (MSP-1<sub>42</sub>). At the time of erythrocyte invasion MSP-1<sub>42</sub> is processed further to a 33 kDa fragment and a 19 kDa C-terminal fragment (MSP-1<sub>19</sub>) (Blackman, et al., 1991,

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Mol. Biochem. Parasitol. 49, 35-44) which is bound to the merozoite surface through an N-glycosylphosphatidyl inositol anchor (GPI) (Haldar, et al., 1985, supra). This second proteolytic cleavage event results in the shedding of the non-covalent associated protein complex from the merozoite surface during invasion. During the invasion process, MSP-1<sub>19</sub> is present on ring forms in the newly invaded erythrocyte (Blackman, et al., 1990, J. Exp. Med. 172, 379-382). The apparent structure of MSP-1<sub>19</sub> is complex, containing 12 cysteines within a span of 100 amino acid residues, and is arranged as two tandem domains that are homologous with epidermal growth factor (EGF) (Blackman, et al., 1991, supra; Morgan et al., 2000, J. Biomol. NMR 17, 337-347). Each putative EGF-domain contains six cyteine residues that would form three disulfide bridges per domain, which force the assembly of several well defined discontinuous epitopes (Farley and Long, 1995, Exp. Parasitol. 80, 328-332; McBride and Heidrich, 1987, supra; Uthaipibull et al, 2001, J. Mol. Biol. 307, 1381-1394).

Because age-dependent development of immunity to malaria is due, at least in part, to antibody against erythrocytic stage parasites (Cohen, S. et al., 1964, Nature 192, 733-737), a malaria vaccine should induce effective antibodies against this developmental stage. Evidence supporting the use of MSP-1<sub>42</sub> and MSP-1<sub>19</sub> in a malaria vaccine is extensive. MSP-1<sub>19</sub>-specific mAbs inhibit *P. falciparum* growth *in vitro* (Blackman et al., 1990, supra) and passively protect mice against infection with *P. yoelii* (Majarian et al., 1984, J. Immunol. 132, 3131-3137; Ling et al., 1994, Parasite Immunol. 16, 63-67). Immunization of *Aotus* monkeys with native *P. falciparum* MSP-1 (Siddiqui, et al.,

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1987, Proc. Natl. Acad. Sci. USA 84, 3014-3018), or *S. cerevisiae* recombinant MSP-1<sub>19</sub> (Kumar et al., 1995, Mol. Med. 1, 325-332; Egan et al., 2000, Infect. Immun. 68, 1418-1427; Stowers et al. 2001, Trends Parasitol. 17, 415-419), protect against a homologous challenge. *E. coli*-expressed *P. yoelii* MSP-1<sub>19</sub> (Burns et al., 1989, J. Immunol. 143, 2670-2676) protects against a homologous challenge in rodent models. Antibodies raised against yeast MSP-1<sub>19</sub> grown in yeast weakly inhibit *Plasmodium* growth *in vitro* (Gozalo et al., 1998, Am. J. Trop. Med. Hyg. 59, 991-997) however this antigen lacks correct structure and induces a strong allergic response (Keitel, W. A., 1999, Vaccine 18, 531-539). MSP-1<sub>19</sub> may not be an optimal vaccine because it does not induce strong T-helper cell responses (Quin et al., 2001, Eur. J. Immunol. 31, 72-81). Poor MSP-1<sub>19</sub> T-cell immunogenicity may be a consequence of its structural stability, which allows it to resist proteolysis, and therefore to resist processing and presentation to the immune system.

Thus, MSP-1<sub>42</sub> may be a better choice as a vaccine candidate (Quin and Langhorne, 2001, Infect. Immun. 69, 2245-2251). Immunization of Aotus monkeys with baculovirus-expressed recombinant MSP-1<sub>42</sub>, protects against a homologous challenge and the anti-sera raised, inhibit *P. falciparum* growth *in vitro*, but do not result in sufficient yield and are not yet available in clinical grade ((Chang et al., 1996, Infect. Immun. 64, 253-261; Chang et al., 1992, J. Immunol. 149, 548-555). Monoclonal antibodies that either protect against infection *in vivo* (Burns et al., 1989, J. Immunol. 143, 2670-2676), or inhibit parasite growth *in vitro* (Blackman et al., 1990, supra), are specific for discontinuous epitopes since they do not react with disulfide-reduced MSP-1<sub>19</sub>

(McBride and Heidrich, 1987, supra; Farley and Long, 1995, Exp. Parasitol. 80, 328-332). Thus, a recombinant vaccine produced from this part of the MSP-1 will require correct disulfide-dependent  
5 conformation to elicit a protective antibody response.

Therefore, heterologous expression of recombinant molecules must replicate the conformation and structure of these proteins to induce an appropriate immune response. Heterologous expression of  
10 recombinant (MSP-1<sub>42</sub>) from eukaryotic expression systems, i.e. baculovirus and yeast, have lead to recombinant molecules that possess post-translational modifications due to N-glycosylation, and are expressed poorly or are misfolded. Post-translational  
15 modification due to N-glycosylation may be problematic for malaria vaccines because malaria parasites lack this ability.

Other attempts at producing MSP-1 in *E. coli* have not produced protective vaccines (Kumar, S. et al.,  
20 1995, Molecular Medicine 1, 325-332) due to problems with endotoxin contamination and possibly to an inability to establish correct disulfide bridging patterns.

Previous attempts show that not only is a good  
25 expression system needed for proper and sufficient expression of MSP-1<sub>42</sub> but, in addition, a good purification protocol is required which removes endotoxin contamination but which retains the proper folding of the antigen for presentation to the immune  
30 system.

#### SUMMARY OF THE INVENTION

The present invention satisfies the needs discussed above. The present invention provides a method for proper expression and purification of the  
35 MSP-1<sub>42</sub> 3D7 allele. The method of the present

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invention results in elimination of contaminating proteins and conservation of the native folding and disulfide bridging of the protein. Therefore, the essentially purified MSP-1<sub>42</sub> protein of the present  
5 invention retains proper conformation for optimal reactivity for vaccine and screening purposes.

Therefore, a major aim of the present invention resides in the production of large amounts of MSP-1<sub>42</sub> which maintain conformational epitopes critical to  
10 epitope formation in pure form (>95% pure) for diagnostic, prophylactic and therapeutic purposes.

This may not seem complicated but, as with most strategies for protein purification, proved to be difficult and unpredictable. *E. coli* was chosen as a  
15 host, even though it had gone out of favor, for two reasons: (1) *E. coli* was known to produce high level of recombinant proteins and (2) recombinant proteins produced in *E. coli* are not glycosylated, which is consistent with the capabilities of malaria parasites.  
20 Several hurdles had to be overcome to achieve the desired expression level in soluble cytoplasmic form which can be sufficiently purified from host cell proteins without sacrificing proper folding of the protein. Problems with *E. coli* endotoxin levels,  
25 antibiotic resistance and the presence of non-MSP-1<sub>42</sub> contaminants had to be resolved.

The final expression construct, pETATPfmSP-1<sub>42</sub>(3D7) (deposited with ATCC under the Budapest Treaty on ----, accession number ----), was the product of a  
30 series of subclonings, with each successive construction reducing the amount of expressed non-MSP-1 sequence. The construction of a DNA vector expressing a *P. falciparum* 3D7 MSP-1<sub>42</sub> molecule proceeded through several steps. A full-length fusion  
35 with *E. coli* thioredoxin at the N-terminus of MSP-1<sub>42</sub>

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was prepared by cloning in the multiple cloning region of the pET32a expression vector (Construct #1, Figure 1A, pET-Trx42). The expressed protein is identified in SEQ ID NO:1. Positive clones were transformed into the highly regulatable T7 RNA polymerase expressing host. Mini-induction experiments were conducted to optimize expression levels of several clones. In these experiments some variables that were investigated included induction temperature, concentration of inducer (IPTG), length of time of induction, and the influence of *E. coli* host background on levels of expression [BL21(DE3) versus AD494 (DE3)]. These variables have been shown to affect the levels of expression and the partitioning of protein in either soluble or insoluble fractions. SDS-PAGE and immunoblotting analysis of crude extracts from cells induced at 37°C showed that the full length fusion, trxA-MSP-1<sub>42</sub> (Construct #1, Figure 1A) comprised greater than 20% of the total *E. coli* protein. However, following cell lysis, all of the fusion protein partitioned into the insoluble fraction and was associated with inclusion bodies. This situation is often the case with heterologous proteins that are expressed at high levels in *E. coli*.

Lowering the culture temperature sequentially from 37°C to 25°C during induction of expression resulted in increasing levels of soluble fusion protein in the post-sonication supernatant. By increasing the soluble protein at this stage, a urea solubilization and refolding step is avoided thereby assuring more native folding of the protein. The post sonication soluble supernatant was applied to a Ni<sup>+2</sup>-NTA agarose affinity column (QIAGEN) and bound protein was eluted with stepwise increasing gradients of imidazole. The expressed thioredoxin-MSP-1<sub>42</sub> fusion

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products from these cells was reactive with mAb 5.2  
(see Table 1) on immunoblots. Our data suggested that  
this expression system would provide sufficient levels  
of recombinant protein for development as a diagnostic  
5 and vaccine antigen, providing proper covalent  
disulfide bridging could be achieved.

A second construct was designed (Construct #2,  
pET(50)MSP-1<sub>42</sub>) to delete the *E. coli* trxA gene  
(thioredoxin protein) from Construct #1 (thioredoxin-  
10 MSP-1<sub>42</sub> fusion). This product was developed as an  
alternative to the full-length thioredoxin fusion to  
address potential FDA regulatory concerns with a  
thioredoxin-MSP-1<sub>42</sub> fusion protein vaccine. The  
product formed retains the His6-tag for affinity  
15 purification and an additional vector encoded sequence  
(approximately 50 amino acids) which include an  
enterokinase cleavage site, and S-peptide tag, and the  
thrombin cleavage site fused to the N-terminus of MSP-  
1<sub>42</sub>. The expressed product is identified in SEQ ID  
20 NO:2. The levels of expression from this construct  
were estimated at approximately 5-10% of the total *E.*  
*coli* protein from crude cell lysates and protein was  
purified to near homogeneity (>85%) with two  
consecutive passes over a Ni<sup>+2</sup>-NTA agarose resin.

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Table 1 - MSP-119-Specific Monoclonal Antibodies

| Antibody   | Immunizing parasite         | Stages | Epitope/location                    | Ref.     |
|------------|-----------------------------|--------|-------------------------------------|----------|
| 2.2-7      | Thai-K1                     | smr*   | Conserved/EGF-like domain 1         | McKay    |
| 12.8-2-1   | Thai-T9-96 (K1 like)        | smr    | Conserved/EGF-like domain 1         | Conway   |
| 5 7.5-1    | Thai-K1                     | smr    | Semi-conserved/EGF-like domain 1    | McBride  |
| 12.10-5-1  | Thai-T9-96 (K1 like)        | smr    | Semi-conserved, EGF-like domain 1&2 | Blackman |
| 5.2        | Uganda-Palo Alto (3D7 like) | smr    | Semi-conserved, EGF-like domain 1   | Chang    |
| 7F1        | Malayan Camp (3D7 like)     | sm     | MSP-133 Strain Specific             | Lyon     |
| Polyclonal | 3D7 MSP-1-42                | smr    | Many epitopes                       | Angov    |

10 \*-schizonts, merozoites, rings

#### References:

Blackman, M.J., et al. 1990, J. Exp. Med. 172:379-382.

Conway, D.J., et al. 1991, Parasitol. 103:1-6.

15 Chang, S.P. et al. 1988, Exp. Parasitol. 67:1-11.

Mackay, M, et al. 1985, EMBO J. 4:3823-3829.

McBride, J.S., et al. 1982, Science, 217:254-257.

Lyon, J.A. et al. 1987, J. Immunology. 138: 895-901.

Polyclonal rabbit MSP-142 antibody was prepared at Walter Reed Army Institute of Research Department of Immunology, by immunization of rabbits with recombinant E. coli-expressed MSP-142 (3D7) adjuvanted with Complete Freund's Adjuvant.

20

Since the levels of expression and apparent protein folding of Construct #2 suggested that a correctly folded non-thioredoxin-fused MSP-1<sub>42</sub> was expressible, a third construct was developed to remove the entire vector non-MSP-1 encoded sequence fused at the N-terminus of Construct #2. This upstream gene sequence was deleted and was replaced with an annealed oligonucleotide linker to regenerate the His6-tag (Construct #3, pET42A). Therefore, Construct #3 contains 9 non-MSP-1<sub>42</sub> amino acids that include the His6 and 3 linker amino acids. The expressed product is identified in SEQ ID NO:3. The non-fused MSP-1<sub>42</sub> molecule from this construct is produced to adequate levels (2-5%) of the total *E. coli* protein and is correctly folded based on immunoreactivity with a series of MSP-1<sub>19</sub> specific mAbs (See Table 1). Potential regulatory concerns over selection in the presence of ampicillin resulted in a final modification on the His6-MSP-1<sub>42</sub> construct (Construct #3) that included the gene for tetracycline (Construct #4, pET-IEGR-MSP142(AT), Figure 1B). Therefore, the plasmid designated as Construct #4 (also His6-MSP-1<sub>42</sub>, but selectable with tetracycline) can be selected in the presence of tetracycline alone during large-scale fermentation or with ampicillin, as necessary. The expressed protein of Construct #4 is identified in SEQ ID NO:3. The final plasmid pET(AT)PfMSP-1<sub>42</sub>(3D7) was created by removing the residual Factor Xa cleavage site. Constructs 1-4 can be used to produce fusion proteins with MSP-1<sub>42</sub> as a source of soluble antigen.

Intensive investigation of variables that affect the efficiency of fermentation and induction of expression were required to optimize His6-MSP-1<sub>42</sub> expression. Some variables which have a significant

effect on target protein yields and bear upon  
purification strategies include the effects of media  
composition, amount of inducer necessary, temperature  
at which inducer is added to the cultures, and length  
5 of time of induction, to name a few.

A low temperature of induction was necessary in  
order to obtain soluble protein. The temperature of  
the cultures had to be reduced from 37°C to about 25°C  
prior to induction. At all higher temperatures,  
10 protein was found in inclusion bodies and difficult to  
isolate. Similarly, we found that the time of  
induction was important for proper and maximal  
expression of the protein. The length of IPTG  
induction was most advantageous at 2-3 hours.  
15 Induction for less time resulted in suboptimal protein  
synthesis and induction for more time resulted in loss  
of product due to lysis and protein degradation.

Cells were suspended in lysis buffer and lysed by  
microfluidization (Microfluidics) in one pass while  
20 the temperature of the sample was maintained below  
10°C at all times to reduce proteolysis. The lysate  
was centrifuged and the pellets and supernates were  
evaluated by immunoblotting.

The clarified lysate was then purified by several  
25 methods. The first method is using the ability of the  
His6-tag sequence expressed as a short N-terminal  
fusion on the target protein to bind to divalent  
cations, i.e. nickel, immobilized onto matrices. The  
w/v cell paste to resin ratio were varied to optimize  
30 yield and purity of the product and to minimize cost.

After elution of the protein from the  $\text{Ni}^{2+}$  chelate  
resin, it is allowed to incubate at 0-4°C overnight to  
promote disulfide bridge formation. This step is  
required because protein disulfide bridge formation  
35 does not occur readily in the cytoplasm of the *E. coli*

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BL21 DE3 expression host due to the reducing nature of this environment. This explains our observation that monoclonal antibodies known to react with properly folded disulfide bridged MSP-1<sub>42</sub> do not react with this protein either in cell lysates, or with partially purified recombinant MSP-1<sub>42</sub> immediately after its elution from the Ni<sup>+2</sup> NTA affinity column. Incubating the eluted protein at 0-4°C for up to 48 hours following the Ni<sup>+2</sup> chromatography promotes proper disulfide bridge formation because after this incubation, all of the monoclonal antibodies now react with the antigen. These data suggest that at the time of lysis and chromatography on the Ni<sup>+2</sup> NTA affinity column the protein is probably folded properly through ionic and hydrophobic interactions, but that the disulfide bridges do not form until the protein is maintained in an oxidative environment over the observed period of time.

The eluted sample was then applied to a Q ion exchange chromatography column. Binding of the sample to the column was checked at varying pH values ranging from pH 6.2 to 9.9. In order to define a pH condition at which the protein would bind to the Q resin, we varied buffer types, e.g. phosphate (pH 6-9) vs. glycine (pH 8.5-11) vs. citrate (pH 4-5-6). Under the final conditions selected, MSP-1<sub>42</sub> partitioned into the flow through and most residual host cell remained proteins bound to the column.

Finally, CM ion exchange chromatography was used to remove low levels of residual *E. coli* protein and endotoxin. Variables optimized included the binding of protein to the column, the salt concentration of the washing and eluting solution.

The purified *P. falciparum* MSP-1<sub>42</sub> was used as a vaccine along with an adjuvant, for example, ADJUVANT

B, and was found to elicit malaria specific antibody responses in monkeys. More importantly, vaccination with the MSP-1<sub>42</sub> elicited neutralizing antibodies and protects monkeys against a malaria infection.

5       Therefore, it is an object of the present invention to provide a recombinant *P. falciparum* MSP-1<sub>42</sub> for use in diagnostic assays and for production of antibodies.

10       It is another object of the present invention to provide compositions comprising purified recombinant *P. falciparum* MSP-1<sub>42</sub>.

15       It is yet another object of the present invention to provide novel vector constructs for recombinantly expressing *P. falciparum* MSP-1<sub>42</sub>, as well as host cells transformed with said vector.

20       It is also an object of the present invention to provide a method for producing and purifying recombinant *P. falciparum* MSP-1<sub>42</sub> protein comprising:  
      growing a host cell containing a vector  
      expressing *P. falciparum* MSP-1<sub>42</sub> proteins in a suitable culture medium,

25       causing expression of said vector sequence as defined above under suitable conditions for production of soluble protein and,  
      lysing said transformed host cells and recovering said MSP-1<sub>42</sub> protein such that it retains its native folding and is essentially free of host toxins.

30       It is also an object of the present invention to provide diagnostic and immunogenic uses of the recombinant *P. falciparum* MSP-1<sub>42</sub> protein of the present invention, as well as to provide kits for diagnostic use for example in malaria screening and confirmatory antibody tests.

35       It is also an object of the present invention to provide monoclonal or polyclonal antibodies, more

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particularly human monoclonal antibodies or mouse monoclonal antibodies which are humanized, which react specifically with MSP-1<sub>42</sub> epitopes, either comprised in peptides or conformational epitopes comprised in recombinant proteins.

It is also an object of the present invention to provide possible uses of anti-MSP-1<sub>42</sub> monoclonal antibodies for malaria antigen detection or for therapy of chronic malaria infection.

It is yet another object of the present invention to provide a malaria vaccine comprising MSP-1<sub>42</sub> of the present invention, in an amount effective to elicit an immune response in an animal against *P. falciparum*; and a pharmaceutically acceptable diluent, carrier, or excipient.

It is another object of the present invention to provide a method for eliciting in a subject an immune response against malaria, the method comprising administering to a subject a composition comprising MSP-1<sub>42</sub> of the present invention. In one aspect of the invention, the DNA vaccine is delivered along with an adjuvant, for example ADJUVANT B.

It is another object of the present invention to provide a method for preventing malaria infection in an animal comprising administering to the animal the MSP-1<sub>42</sub> of the present invention.

The vaccine according to the present invention is inherently safe, is not painful to administer, and should not result in adverse side effects to the vaccinated individual.

The present invention also provides vectors for the production of a recombinant MSP-1<sub>42</sub>, host cells containing the vectors, a method for fermenting and inducing the host cells, and a method for isolating and purifying the recombinant protein. Also provided

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is a method for bulk fermentation and expression of MSP-1<sub>42</sub>.

All the objects of the present invention are considered to have been met by the embodiments  
5 as set out below.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1A: pET32a plasmid map.

Figure 1B: pET(AT)PfMSP-1<sub>42</sub> plasmid map.

Figure 2A, 2B and 2C: SDS-PAGE analysis of MSP-1<sub>42</sub>  
10 during Purification and of the Final Product (FMP1). Protein detected by Coomassie Blue staining (2A and 2B) and immunoblotting (2C). 2A, non-reduced samples: lane 1, nickel chelate eluate; lane 2, Q flow through; lane 3 CM eluate. 2B, 10 ug FMP1 electrophoresed under  
15 non-reduced (lane 1) or reduced (lane 2) conditions. 2C, immunoblotting of 1 ug of FMP electrophoresed under non-reducing: lane 1 mAb 7F1; lane 2, mAb 12.10; lane 3, rabbit anti-*E. coli* antiserum.

Figure 3: FMP1 Stability. Commassie Blue stained  
20 non-reduced SDS-PAGE gel with 10 ug samples of FMP1 stored for 18 months under various conditions. Lane 1, -80°C; lane 2, -20°C; lane 3, 4°C.

Figure 4: FMP1 Immunogenicity. Rhesus monkeys were boosted 1, 3, 5, and 7 months after priming with  
25 FMP1/ADJUVANT B (rectangles) or FMP1/alum (circles). Sera were collected just prior to immunization (arrows) and two weeks after each immunization. In the case of the ADJUVANT B cohort serum collection continued monthly for 9 months. Antibody titers were  
30 measured by IFA (filled symbols) and kinetic ELISA (open symbols).

Figure 5: Merozoite Invasion Inhibition. Triplicate synchronous cultures of *P. falciparum* schizont-infected erythrocytes were incubated for 24  
35 hours with the IgG fractions of pre-immune (open

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diamonds) or immune (filled diamonds) IgG fractions from rabbits immunized with FMP1 in Fruends adjuvents. IgG was tested at doses of 200, 400, and 800 ug/ml and parasite growth was quantified by counting 5000  
 5 erythroctyes or 100 parasitized erythrocytes, whichever occurred first. Data are shown as the mean parasitemia and 95% confidence interval.

Figure 6: CMI responses to FMP1/ADJUVANT B. Peripheral blood mononuclear cells (PBMC) were  
 10 stimulated with FMP1 (top) or *P. falciparum* parasitized erythrocytes (PBRC, bottom) and proliferation was measured by 3H-thymidine uptake. Each panel contains data from all individuals within a vaccine group and individuals are differentiated by  
 15 symbol. Samples were collected prior to immunization (pre) and two weeks following each immunization (post 1, post 2, post 3).

#### DETAILED DESCRIPTION

In the description that follows, a number of  
 20 terms used in recombinant DNA, parasitology and immunology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are  
 25 provided.

In general, an 'epitope' is defined as a linear array of 3-10 amino acids aligned along the surface of a protein. In a linear epitope, the amino acids are joined sequentially and follow the primary structure  
 30 of the protein. In a conformational epitope, residues are not joined sequentially, but lie linearly along the surface due to the conformation (folding) of the protein. With respect to conformational epitopes, the length of the epitope-defining sequence can be subject  
 35 to wide variations. The portions of the primer

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structure of the antigen between the residues defining the epitope may not be critical to the structure of the conformational epitope. For example, deletion or substitution of these intervening sequences may not affect the conformational epitope provided sequences critical to epitope conformation are maintained (e.g. cysteines involved in disulfide bonding, glycosylation sites, etc.). A conformational epitope may also be formed by 2 or more essential regions of subunits of a homo-oligomer or hetero-oligomer. As used herein, 'epitope' or 'antigenic determinant' means an amino acid sequence that is immunoreactive. As used herein, an epitope of a designated polypeptide denotes epitopes with the same amino acid sequence as the epitope in the designated polypeptide, and immunologic equivalents thereof. Such equivalents also include strain, subtype (=genotype), or type(group)-specific variants, e.g. of the currently known sequences or strains belonging to *Plasmodium* such as 3D7, FVO and CAMP, or any other known or newly defined *Plasmodium* strain.

The term 'solid phase' intends a solid body to which the individual *P. falciparum* antigen is bound covalently or by noncovalent means such as hydrophobic, ionic, or van der Waals association.

The term 'biological sample' intends a fluid or tissue of a mammalian individual (e.g. an anthropoid, a human), reptilian, avian, or any other zoo or farm animal that commonly contains antibodies produced by the individual, more particularly antibodies against malaria. The fluid or tissue may also contain *P. falciparum* antigen. Such components are known in the art and include, without limitation, blood, plasma, serum, urine, spinal fluid, lymph fluid, secretions of the respiratory, intestinal or genitourinary tracts,

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tears, saliva, milk, white blood cells and myelomas. Body components include biological liquids. The term 'biological fluid' refers to a fluid obtained from an organism. Some biological fluids are used as a source of other products, such as clotting factors (e.g. Factor VIII;C), serum albumin, growth hormone and the like.

The term 'immunologically reactive' means that the antigen in question will react specifically with anti-MSP-1 antibodies present in a body component from a malaria infected individual.

The term 'immune complex' intends the combination formed when an antibody binds to an epitope on an antigen.

The term 'MSP-1<sub>42</sub>' as used herein refers to the polymorphic C-terminal 42 kDa protein fragment or polypeptide resulting from the processing by proteases of the 195 kDa membrane-anchored MSP-1 precursor. During merozoite invasion, the 42 kDa fragment is subjected to secondary processing producing a 33-kDa fragment (MSP-1<sub>33</sub>) and a 19 kDa C-terminal fragment, (MSP-1<sub>19</sub>) which remains attached via GPI to the surface of the invading merozoite. The MSP-1<sub>42</sub> protein extends from approximately amino acid (aa) 1327 to about aa 1700 of the full-length precursor protein (Genbank accession #Z35327).

The term 'MSP-1<sub>42</sub>' as used herein also includes analogs and truncated forms that are immunologically cross-reactive with natural MSP-1<sub>42</sub>. By 'MSP-1<sub>42</sub>' is intended MSP-1<sub>42</sub> from other strains of *Plasmodium*, or any other newly identified strain of *Plasmodium*.

The term 'homo-oligomer' as used herein refers to a complex of MSP-1<sub>42</sub> containing more than one MSP-1<sub>42</sub> monomer, e.g. MSP-1<sub>42</sub>/MSP-1<sub>42</sub> dimers, trimers or

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tetramers, or any higher-order homo-oligomers of MSP-1<sub>42</sub> are all 'homo-oligomers' within the scope of this definition. The oligomers may contain one, two, or several different monomers of MSP-1<sub>42</sub> obtained from different strains of *Plasmodium falciparum* including for example 3D7, Camp, FVO, and others. Such mixed oligomers are still homo-oligomers within the scope of this invention, and may allow more universal diagnosis, prophylaxis or treatment of malaria.

10       The term 'purified' as applied to proteins herein refers to a composition wherein the desired protein comprises at least 35% of the total protein component in the composition. The desired protein preferably comprises at least 40%, more preferably at least about 50%, more preferably at least about 60%, still more preferably at least about 70%, even more preferably at least about 80%, even more preferably at least about 90%, and most preferably at least about 95% of the total protein component. The composition may contain other compounds such as carbohydrates, salts, lipids, solvents, and the like, without affecting the determination of the percentage purity as used herein. An 'isolated' MSP-1<sub>42</sub> protein intends a *Plasmodium* protein composition that is at least 35% pure.

25       The term 'essentially purified proteins' refers to proteins purified such that they can be used for *in vitro* diagnostic methods and as a prophylactic compound. These proteins are substantially free from cellular proteins, vector-derived proteins or other *Plasmodium* components. The proteins of the present invention are purified to homogeneity, at least 80% pure, preferably, 90%, more preferably 95%, more preferably 97%, more preferably 98%, more preferably 99%, even more preferably 99.5%.

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The term 'recombinantly expressed' used within the context of the present invention refers to the fact that the proteins of the present invention are produced by recombinant expression methods be it in  
5 prokaryotes, or lower or higher eukaryotes as discussed in detail below.

The term 'lower eukaryote' refers to host cells such as yeast, fungi and the like. Lower eukaryotes are generally (but not necessarily) unicellular.  
10 Preferred lower eukaryotes are yeasts, particularly species within *Saccharomyces*. *Schizosaccharomyces*, *Kluveromyces*, *Pichia* (e.g. *Pichia pastoris*), *Hansenula* (e.g. *Hansenula polymorpha*, *Yarrowia*, *Schwaniomyces*, *Schizosaccharomyces*, *Zygosaccharomyces* and the like.  
15 *Saccharomyces cerevisiae*, *S. carlsberoensis* and *K. lactis* are the most commonly used yeast hosts, and are convenient fungal hosts.

The term 'prokaryotes' refers to hosts such as *E.coli*, *Lactobacillus*, *Lactococcus*, *Salmonella*,  
20 *Streptococcus*, *Bacillus subtilis* or *Streptomyces*. Also these hosts are contemplated within the present invention.

The term 'higher eukaryote' refers to host cells derived from higher animals, such as mammals,  
25 reptiles, insects, and the like. Presently preferred higher eukaryote host cells are derived from Chinese hamster (e.g. CHO), monkey (e.g. COS and Vero cells), baby hamster kidney (BHK), pig kidney (PK15), rabbit kidney 13 cells (RK13), the human osteosarcoma cell  
30 line 143 B, the human cell line HeLa and human hepatoma cell lines like Hep G2, and insect cell lines (e.g. *Spodoptera frugiperda*). The host cells may be provided in suspension or flask cultures, tissue cultures, organ cultures and the like. Alternatively  
35 the host cells may also be transgenic animals.

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The term 'polypeptide' refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not refer to or exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogues of an amino acid (including, for example, unnatural amino acids, PNA, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

The term 'recombinant polynucleotide or nucleic acid' intends a polynucleotide or nucleic acid of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation : (1) is not associated with all or a portion of a polynucleotide with which it is associated in nature, (2) is linked to a polynucleotide other than that to which it is linked in nature, or (3) does not occur in nature.

The term 'recombinant host cells', 'host cells', 'cells', 'cell lines', 'cell cultures', and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be or have been, used as recipients for a recombinant vector or other transfer polynucleotide, and include the progeny of the original cell which has been transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as

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the original parent, due to natural, accidental, or deliberate mutation.

The term `replicon` is any genetic element, e.g., a plasmid, a chromosome, a virus, a cosmid, etc., that  
5 behaves as an autonomous unit of polynucleotide replication within a cell; i.e., capable of replication under its own control.

The term `vector` is a replicon further comprising sequences providing replication and/or  
10 expression of a desired open reading frame.

The term `control sequence` refers to polynucleotide sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs  
15 depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and terminators; in eukaryotes, generally, such control sequences include promoters, terminators and, in some instances,  
20 enhancers. The term `control sequences` is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous, for example, leader sequences which govern secretion.

The term `promoter` is a nucleotide sequence which is comprised of consensus sequences which allow the binding of RNA polymerase to the DNA template in a manner such that mRNA production initiates at the normal transcription initiation site for the adjacent  
30 structural gene.

The expression `operably linked` refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence `operably linked`  
35 to a coding sequence is ligated in such a way that

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expression of the coding sequence is achieved under conditions compatible with the control sequences.

An 'open reading frame' (ORF) is a region of a polynucleotide sequence which encodes a polypeptide and does not contain stop codons; this region may represent a portion of a coding sequence or a total coding sequence.

A 'coding sequence' is a polynucleotide sequence which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include but is not limited to mRNA, DNA (including cDNA), and recombinant polynucleotide sequences.

The term 'immunogenic' refers to the ability of a substance to cause a humoral and/or cellular response, whether alone or when linked to a carrier, in the presence or absence of an adjuvant. 'Neutralization' refers to an immune response that blocks the infectivity, either partially or fully, of an infectious agent. A 'vaccine' is an immunogenic composition capable of eliciting protection against malaria, whether partial or complete. A vaccine may also be useful for treatment of an infected individual, in which case it is called a therapeutic vaccine.

The term 'therapeutic' refers to a composition capable of treating malaria infection. The term 'effective amount' for a therapeutic or prophylactic treatment refers to an amount of epitope-bearing polypeptide sufficient to induce an immunogenic response in the individual to which it is administered, or to otherwise detectably immunoreact

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in its intended system (e.g., immunoassay).  
Preferably, the effective amount is sufficient to  
effect treatment, as defined above. The exact amount  
necessary will vary according to the application. For  
5 vaccine applications or for the generation of  
polyclonal antiserum/antibodies, for example, the  
effective amount may vary depending on the species,  
age, and general condition of the individual, the  
severity of the condition being treated, the  
10 particular polypeptide selected and its mode of  
administration, etc. It is also believed that  
effective amounts will be found within a relatively  
large, non-critical range. An appropriate effective  
amount can be readily determined using only routine  
15 experimentation. Preferred ranges of MSP-1<sub>42</sub> for  
prophylaxis of malaria disease are about 0.01 to 1000  
ug/dose, more preferably about 0.1 to 100 ug/dose,  
most preferably about 10-50 ug/dose. Several doses may  
be needed per individual in order to achieve a  
20 sufficient immune response and subsequent protection  
against malaria.

More particularly, the present invention  
contemplates essentially purified MSP-1<sub>42</sub> and a method  
25 for isolating or purifying recombinant MSP-1<sub>42</sub>  
protein, characterized in that upon lysing the  
transformed host cells to isolate the recombinantly  
expressed protein, the disulfide bonds necessary for  
proper folding of the protein are preserved.

30 The term 'MSP-1<sub>42</sub>' refers to a polypeptide or an  
analogue thereof (e.g. mimotopes) comprising an amino  
acid sequence (and/or amino acid analogues) defining  
at least one MSP-1<sub>42</sub> epitope. Typically, the sequences  
defining the epitope correspond to the amino acid  
35 sequence of MSP-1<sub>42</sub> region of *P. falciparum* (either

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identically or via substitution of analogues of the native amino acid residue that do not destroy the epitope). The MSP-1<sub>42</sub> protein corresponds to a nucleotide sequence identified in SEQ ID NO:5 and spans from amino acid 1327 to 1701 of MSP-1 3D7 allele (SEQ ID NO:6). Upon expression in the parasite system (non-glycosylated), it is believed to have an approximate molecular weight of 42 kDa as determined by SDS-PAGE. It is understood that these protein endpoints are approximations (e.g. the carboxy terminal end of MSP-1<sub>42</sub> could lie somewhere in the 1700 to 1720 amino acid region). The absolute C-terminus is not defined due to the post-translational modification that transfers MSP-1 to a GPI lipid membrane anchor.

The MSP-1<sub>42</sub> antigen used in the present invention is preferably a full-length protein, or a substantially full-length version, i.e. containing functional fragments thereof (e.g. fragments which are not missing sequence essential to the formation or retention of an epitope). Furthermore, the *P. falciparum* antigen of the present invention can also include other sequences that do not block or prevent the formation of the conformational epitope of interest. The presence or absence of a conformational epitope can be readily determined through screening the antigen of interest with an antibody as described in the Examples below (polyclonal serum or monoclonal to the conformational epitope) and comparing its reactivity to that of a denatured version of the antigen which retains only linear epitopes (if any).

The *P. falciparum* antigen of the present invention can be made by any recombinant method that provides the epitope of interest. For example, recombinant expression in *E. coli* is a preferred

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method to provide non-glycosylated antigens in  
'native' conformation. This is most desirable because  
natural *P. falciparum* antigens are not glycosylated.  
Proteins secreted from mammalian cells may contain  
5 modifications including galactose or sialic acids  
which may be undesirable for certain diagnostic or  
vaccine applications. However, it may also be possible  
and sufficient for certain applications, as it is  
known for proteins, to express the antigen in other  
10 recombinant hosts such as baculovirus and yeast or  
higher eukaryotes, as long as glycosylation is  
inhibited.

The proteins according to the present invention  
may be secreted or expressed within compartments of  
15 the cell. Preferably, however, the proteins of the  
present invention are expressed within the cell and  
are released upon lysing the cells.

It is also understood that the isolates used in  
the examples section of the present invention were not  
20 intended to limit the scope of the invention and that  
an equivalent sequence from a *P. falciparum* isolate  
from another allele, e.g. FVO, or CAMP, can be used to  
produce a recombinant MSP-1<sub>42</sub> protein using the methods  
described in the present application. Other new  
25 strains of *Plasmodium* may be a suitable source of MSP-  
1<sub>42</sub> sequence for the practice of the present invention.

The MSP-1<sub>42</sub> protein of the present invention is  
expressed as part of a recombinant vector. The  
present invention relates more particularly to the  
30 MSP-1<sub>42</sub> nucleic acid sequence in recombinant nucleic  
acids pETATpfMSP-1<sub>42</sub>(3D7) as represented in SEQ ID NO:7  
or parts thereof. The MSP-1<sub>42</sub> genomic sequence was  
cloned into pET32a from Novagen (Madison, Wisconsin).  
This plasmid comprises, in sequence, a T7 promoter,  
35 optionally a lac operator, a ribosome binding site,

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restriction sites to allow insertion of the structural gene and a T7 terminator sequence. Other vectors provided include pET-Trx42, pET(50)MSP-1<sub>42</sub>, pET42A, pET-IEGR-MSP-1<sub>42</sub>(AT) all described below in Materials and Methods. Examples of other plasmids which contain the T7 inducible promoter include the expression plasmids pET-17b, pET-11a, pET-24a-d(+), and pEt-9a, all from Novagen (Madison, Wisconsin); see the Novagen catalogue.

10       The present invention also contemplates host cells transformed with a recombinant vector as defined above. In a preferred embodiment, *E. coli* strain BL21 (DE3) (F-ompT hsdSB(rB-mB-) gal dcm (DE3)) is employed. The above plasmids may be transformed into 15 this strain or other strains of *E. coli* having the following characteristics: a T7 RNA polymerase rec gene, Lon, ompT protease mutants or any other *E. coli* with a protease deficiency such as *E. coli* origami. Preferably, the host includes BL21(DE3) and any of its 20 precursors. Other host cells such as insect cells can be used taking into account that other cells may result in lower levels of expression.

25       Eukaryotic hosts include lower and higher eukaryotic hosts as described in the definitions section. Lower eukaryotic hosts include yeast cells well known in the art. Higher eukaryotic hosts mainly include mammalian cell lines known in the art and include many immortalized cell lines available from the ATCC, including HeLa cells, Chinese hamster ovary 30 (CHO) cells, Baby hamster kidney (BHK) cells, PK15, RK13 and a number of other cell lines. MSP-1<sub>42</sub> expressed in these cells will be glycosylated unless the cells have been altered such that glycosylation of the recombinant protein is not possible. It is 35 expected that when producing MSP-1<sub>42</sub> in a eukaryotic

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expression system, extensive investigation into methods for expressing, isolating, purifying, and characterizing the protein would be required as eukaryotic cells post-translationally modify this protein and this would alter protein structure and immunogenicity.

Methods for introducing vectors into cells are known in the art. Please see e.g., Maniatis, Fitch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. Host cells provided by this invention include *E. coli* containing pET-Trx42, *E. coli* containing pET(50)MSP-1<sub>42</sub>, and *E. coli* containing pET42A, *E. coli* containing pET-IEGR-MSP-1<sub>42</sub>(AT), and *E. coli* containing pET(AT)PfMSP-1<sub>42</sub>(3D7).

A preferred method for isolating or purifying MSP-1<sub>42</sub> as defined above is further characterized as comprising at least the following steps:

- (i) growing a host cell as defined above transformed with a recombinant vector expressing MSP-1<sub>42</sub> proteins in a suitable culture medium,
- (ii) causing expression of said vector sequence as defined above under suitable conditions for production of a soluble protein,
- (iii) lysing said transformed host cells and recovering said MSP-1<sub>42</sub> protein such that it retains its native conformation and is essentially pure.

Once the host has been transformed with the vector, the transformed cells are grown in culture in the presence of the desired antibiotic. For FDA regulatory purposes, it is preferable to use tetracycline or kanamycin. When cells reach optimal biomass density, in this case about 0.4 OD 600 in

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small culture flasks or 4-6 OD 600 in bulk fermentors, the cells are induced to produce the recombinant protein. The inventors have found after trial and error that for expression of a soluble MSP-1<sub>42</sub>, it was  
5 necessary to cool the culture to a range of about 10°C - 20°C, more preferably about 15°C - 28°C, most preferably about 24 to 26°C prior to induction. The concentration of inducer, i.e. IPTG, added affects the maximal protein synthesis. It was found that a  
10 concentration of 0.1 mM IPTG was best, however, a range of 0.05 to 0.5 mM would be sufficient to produce 80-100% of maximal.

The cells were then collected and lysed to release the recombinant protein. Preferably, lysis  
15 should occur at a paste to buffer ratio of 1:3 w/v to reduce viscosity and volume of sample loaded on Ni-NTA column. Preferably, lysis is in the presence of imidazole which reduces non specific binding of *E. coli* protein to Ni resin, and benzonase which is able  
20 to digest *E. coli* nucleic acids at a reduced temperature. Lysis is preferably at a temperature of about 0°C - 24°C, more preferably about 5-15°C in order to retain native folding of the MSP-1<sub>42</sub> protein and to reduce proteolysis. A high salt concentration  
25 of about 0.5-1.0 M is preferable. Salts used include NaCl or other monovalent ions.

Preferably, the *E. coli* endotoxin is separated and removed from the recombinant protein. This can be done several ways. For MSP-1<sub>42</sub>, endotoxin was removed  
30 by applying to a Ni<sup>2+</sup>-NTA column. The removal of endotoxin depended on washing at low pH, about 5.8 to 6.5, preferably about pH 6.2, in high salt, about 0.5 to about 1.0 mM, preferably about 500 mM NaCl at a flow rate of about 20 to about 35 ml/min, preferably  
35 about 30 ml/min. The cell paste to resin ratio can be

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about 5:1 to about 7:1 w/v, preferably about 6:1 w/v. The recombinant protein can be eluted by addition of high pH buffer of about 7.5 to about 8.5, preferably about pH 8.0, in a phosphate buffer of about 10-20 mM, more preferably about 10 mM sodium phosphate buffer.

At this point the recombinant protein is about 50% pure. If further purity is required, ion-exchange chromatography can be utilized. The column is preferably with an ionic character such that a pH to reduce protein binding and promote endotoxin and nucleic acid binding can be used.

Finally, the flow through sample (about 0.1 mg/ml), can be subjected to further ion exchange chromatography for further concentration and purification. The MSP-1<sub>42</sub> of the present invention was subjected to CM ion exchange chromatography. The pH of the buffer can be about 5.2 to about 6.2, preferably about 6.0. The salt concentration is about 25 mM to about 50 mM, preferably about 35 mM.

The bulk process for the isolation of purified MSP-1<sub>42</sub> differs little from the process described above. The concentration of imidazole is changed to about 120 mM to about 200 mM, preferably about 160 mM in order to specifically elute MSP-1<sub>42</sub>.

The present invention further relates to a composition comprising at least one of the following

MSP-1<sub>42</sub> peptides as listed in Table 3:

MSP-1<sub>42</sub> alone (SEQ ID NO:5) spanning amino acids to 1326-1701 of MSP-1,

MSP-1<sub>42</sub> with thioredoxin from vector pET-Trx 42 (SEQ ID NO:1);

MSP-1<sub>42</sub> without thioredoxin from vector pET(5)MSP-1<sub>42</sub> (SEQ ID NO:2);

MSP-1<sub>42</sub> plus His6tag produced from vector pET42A (SEQ ID NO:3)

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MSP-1<sub>42</sub> without Factor Xa from vector pET-IEGR-MSP-1<sub>42</sub> (AT) (SEQ ID NO:3);

MSP-1<sub>42</sub> plus 17 amino acids at N-terminal in final construct (SEQ ID NO :3), from construct pET(AT)PfMSP-1<sub>42</sub>(3D7), the final expressed product referred to as FMP-1 (SEQ ID NO:7).

The present invention also relates to a composition comprising peptides or polypeptides as described above, for *in vitro* detection of malaria antibodies present in a biological sample.

The present invention also relates to a composition comprising at least one of the following MSP-1<sub>42</sub> conformational epitopes:

epitope recognized by monoclonal antibodies 12.10, 12.8, 7.5, 2.2, 1E1 (Blackman et al., 1990, supra; Conway et al., 1991, Parasitology 103,1-6; McBride et al., 1982, Science 217, 254-257; Mackay et al., 1985, Embo J. 4, 3823-3829).

epitope recognized by monoclonal antibody 5.2 (Chang et al., 1988, Exp. Parasitol. 67, 1-11), and epitope recogized by monoclonal antibody 7F1 (Lyon et al., 1987, J. Immunol. 138, 895-901).

The present invention also relates to an MSP-1<sub>42</sub> specific antibody raised upon immunizing an animal with a peptide or protein composition, with said antibody being specifically reactive with any of the polypeptides or peptides as defined above, and with said antibody being preferably a monoclonal antibody.

The present invention also relates to an MSP-1<sub>42</sub> specific antibody screened from a variable chain library in plasmids or phages or from a population of human B-cells by means of a process known in the art, with said antibody being reactive with any of the polypeptides or peptides as defined above, and with said antibody being preferably a monoclonal antibody.

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The MSP-1<sub>42</sub> specific monoclonal antibodies of the invention can be produced by any hybridoma liable to be formed according to classical methods from splenic or lymph node cells of an animal, particularly from a mouse or rat, immunized against the *Plasmodium* polypeptides or peptides according to the invention, as defined above on the one hand, and of cells of a myeloma cell line on the other hand, and to be selected by the ability of the hybridoma to produce the monoclonal antibodies recognizing the polypeptides which has been initially used for the immunization of the animals.

The antibodies involved in the invention can be labelled by an appropriate label of the enzymatic, fluorescent, or radioactive type.

The monoclonal antibodies according to this preferred embodiment of the invention may be humanized versions of mouse monoclonal antibodies made by means of recombinant DNA technology, departing from parts of mouse and/or human genomic DNA sequences coding for H and L chains from cDNA or genomic clones coding for H and L chains.

Alternatively the monoclonal antibodies according to this preferred embodiment of the invention may be human monoclonal antibodies. These antibodies according to the present embodiment of the invention can also be derived from human peripheral blood lymphocytes of patients infected with malaria, or vaccinated against malaria. Such human monoclonal antibodies are prepared, for instance, by means of human peripheral blood lymphocytes (PBL) repopulation of severe combined immune deficiency (SCID) mice, or by means of transgenic mice in which human immunoglobulin genes have been used to replace the mouse genes.

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The invention also relates to the use of the proteins or peptides of the invention, for the selection of recombinant antibodies by the process of repertoire cloning.

5       Antibodies directed to peptides or single or specific proteins derived from a certain strain may be used as a medicament, more particularly for incorporation into an immunoassay for the detection of *Plasmodium* strains for detecting the presence of MSP-  
10   1<sub>42</sub> antigens, or antigens containing MSP-1<sub>42</sub> epitopes, for prognosing/monitoring of malaria disease, or as therapeutic agents.

          Alternatively, the present invention also relates to the use of any of the above-specified MSP-1<sub>42</sub>  
15   monoclonal antibodies for the preparation of an immunoassay kit for detecting the presence of MSP-1<sub>42</sub> antigen or antigens containing MSP-1<sub>42</sub> epitopes in a biological sample, for the preparation of a kit for prognosing/monitoring of malaria disease or for the  
20   preparation of a malaria medicament.

The present invention also relates to a method for *in vitro* diagnosis or detection of malaria antigen present in a biological sample, comprising at least the following steps:

25       (i) contacting said biological sample with any of the MSP-1<sub>42</sub> specific monoclonal antibodies as defined above, preferably in an immobilized form under appropriate conditions which allow the formation of an immune complex,

30       (ii) removing unbound components,

          (iii) incubating the immune complexes formed with heterologous antibodies, which specifically bind to the antibodies present in the sample to be analyzed, with said heterologous antibodies conjugated to a  
35   detectable label under appropriate conditions,

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(iv) detecting the presence of said immune complexes visually or mechanically (e.g. by means of densitometry, fluorimetry, colorimetry).

The present invention also relates to a kit for  
5 *in vitro* diagnosis of a malaria antigen present in a biological sample, comprising:

at least one monoclonal antibody as defined above, with said antibody being preferentially immobilized on a solid substrate,

10 a buffer or components necessary for producing the buffer enabling binding reaction between these antibodies and the malaria antigens present in the biological sample, and

15 a means for detecting the immune complexes formed in the preceding binding reaction.

The kit can possibly also include an automated scanning and interpretation device for inferring the malaria antigens present in the sample from the observed binding pattern.

20 Monoclonal antibodies according to the present invention are suitable both as therapeutic and prophylactic agents for treating or preventing malaria infection in susceptible malaria-infected subjects. Subjects include rodents such as mice or guinea pigs,  
25 monkeys, and other mammals, including humans.

In general, this will comprise administering a therapeutically or prophylactically effective amount of one or more monoclonal antibodies of the present invention to a susceptible subject or one exhibiting  
30 malaria infection. Any active form of the antibody can be administered, including Fab and F(ab')<sub>2</sub> fragments. Antibodies of the present invention can be produced in any system, including insect cells, baculovirus expression systems, chickens, rabbits,  
35 goats, cows, or plants such as tomato, potato, banana

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or strawberry. Methods for the production of antibodies in these systems are known to a person with ordinary skill in the art. Preferably, the antibodies used are compatible with the recipient species such  
 5 that the immune response to the MAbs does not result in clearance of the MAbs before parasite can be controlled, and the induced immune response to the MAbs in the subject does not induce "serum sickness" in the subject. Preferably, the MAbs administered  
 10 exhibit some secondary functions such as binding to Fc receptors of the subject.

Treatment of individuals having malaria infection may comprise the administration of a therapeutically effective amount of MSP-1<sub>42</sub> antibodies of the present  
 15 invention. The antibodies can be provided in a kit as described below. The antibodies can be used or administered as a mixture, for example in equal amounts, or individually, provided in sequence, or administered all at once. In providing a patient with  
 20 antibodies, or fragments thereof, capable of binding to MSP-1<sub>42</sub>, or an antibody capable of protecting against malaria in a recipient patient, the dosage of administered agent will vary depending upon such factors as the patient's age, weight, height, sex,  
 25 general medical condition, previous medical history, etc.

In general, it is desirable to provide the recipient with a dosage of antibody which is in the range of from about 1 pg/kg-100 pg/kg, 100 pg/kg-500  
 30 pg/kg, 500 pg/kg-1 ng/kg, 1 ng/kg-100 ng/kg, 100 ng/kg-500 ng/kg, 500 ng/kg- 1 ug/kg, 1 ug/kg- 100 ug/kg, 100 ug/kg-500 ug/kg, 500 ug/kg- 1 mg/kg, 1 mg/kg-50 mg/kg, 50 mg/kg-100 mg/kg, 100 mg/kg-500 mg/kg, 500 mg/kg-1 g/kg, 1 g/kg-5 g/kg, 5 g/kg-10 g/kg

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(body weight of recipient), although a lower or higher dosage may be administered.

In a similar approach, another prophylactic use of the monoclonal antibodies of the present invention is the active immunization of a patient using an anti-idiotypic antibody raised against one of the present monoclonal antibodies. Immunization with an anti-idiotypic which mimics the structure of the epitope could elicit an active anti-MSP-1<sub>42</sub> response (Linthicum, D.S. and Farid, N.R., Anti-Idiotypes, Receptors, and Molecular Mimicry (1988), pp 1-5 and 285-300).

Likewise, active immunization can be induced by administering one or more antigenic and/or immunogenic epitopes as a component of a subunit vaccine. Vaccination could be performed orally or parenterally in amounts sufficient to enable the recipient to generate protective antibodies against this biologically functional region, prophylactically or therapeutically. The host can be actively immunized with the antigenic/immunogenic peptide in pure form, a fragment of the peptide, or a modified form of the peptide. One or more amino acids, not corresponding to the original protein sequence can be added to the amino or carboxyl terminus of the original peptide, or truncated form of peptide. Such extra amino acids are useful for coupling the peptide to another peptide, to a large carrier protein, or to a support. Amino acids that are useful for these purposes include: tyrosine, lysine, glutamic acid, aspartic acid, cyteine and derivatives thereof. Alternative protein modification techniques may be used e.g., NH<sub>2</sub>-acetylation or COOH-terminal amidation, to provide additional means for coupling or fusing the peptide to another protein or peptide molecule or to a support.

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The antibodies capable of protecting against malaria are intended to be provided to recipient subjects in an amount sufficient to effect a reduction in the malaria infection symptoms. An amount is said to be sufficient to "effect" the reduction of infection symptoms if the dosage, route of administration, etc. of the agent are sufficient to influence such a response. Responses to antibody administration can be measured by analysis of subject's vital signs.

The present invention more particularly relates to a composition comprising at least one of the above-specified peptides or a recombinant MSP-1<sub>42</sub> protein composition as defined above, for use as a vaccine for immunizing a mammal, preferably humans, against malaria, comprising administering a sufficient amount of the composition possibly accompanied by pharmaceutically acceptable adjuvant(s), to produce an immune response.

Immunogenic compositions can be prepared according to methods known in the art. The present compositions comprise an immunogenic amount of a recombinant MSP-1<sub>42</sub> proteins or peptides as defined above, usually combined with a pharmaceutically acceptable carrier, preferably further comprising an adjuvant.

The proteins of the present invention, preferably purified MSP-1<sub>42</sub> derived from pETATPfMBP-1<sub>42</sub> (3D7) or FMP-1, are expected to provide a particularly useful vaccine antigen, since the antigen is able to induce invasion inhibitory antibodies as well as high titer antibodies that react with schizont-infected erythrocytes.

Pharmaceutically acceptable carriers include any carrier that does not itself induce the production of

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antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, 5 polymeric amino acids, amino acid copolymers; and inactive virus particles. Such carriers are well known to those of ordinary skill in the art.

Preferred adjuvants to enhance effectiveness of the composition include adjuvants listed below with 10 formulation for one human vaccine dose of 0.5 ml. the dosages of components described below are preferred dosages; variations in dosages that do not affect the function of the adjuvant are also encompassed by the present invention as can be readily determined by the 15 person skilled in the art.

Adjuvant A, described in WO 96/33739, with the formulation 0.25 mg cholesterol, 1 mg dioleoyl phosphotidylcholine, 50 ug 3D-MPL, and 50 ug QS21 and consisting of small liposomes wherein the QS21 and the 20 3D-MPL are in the membranes of the liposomes;

Adjuvant B, described in U.S. Patent no. 6,146,632, with the formulation 10.68 mg squalene, 11.86 mg tocopherol, 4.85 mg Tween 80, 50 ug 3D-MPL, and 50 ug QS21 and consisting of an oil-in water 25 emulsion comprising the squalene and alpha-tocopherol, the emulsion being in admixture with the QS21 and 3-DPML;

Adjuvant C, described in WO 96/33739, with the formulation 0.25 mg cholesterol, 1 mg dioleoyl 30 phosphotidylcholine, 50 ug 3D-MPL, 50 ug QS21 and 0.5 mg  $\text{AlOH}_3$  and consisting of small liposomes wherein the saponin (QS21) and the LPS-derivative (3D-MPL) are in the membranes of the liposomes and wherein the liposomes and the antigen are absorbed onto a metallic 35 salt particle carrier ( $\text{AlOH}_3$ );

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Adjuvant D, with the formulation 0.5 mg  $\text{AlOH}_3$ ,  
500 ug of unmethylated immunostimulatory  
oligonucleotide CpG described in WO 96/02555 (CpG =  
5'-tcg tcg ttt tgt cgt ttt gtc gtt) (SEQ ID NO:8)

5 where antigen and immunostimulant (CpG) are absorbed  
onto a metallic salt particle carrier ( $\text{AlOH}_3$ );

Adjuvant E, described in WO 96/33739, with the  
formulation 0.25 mg cholesterol, 1 mg dioleoyl  
phosphatidylcholine, 50 ug QS21, and 0.5 mg  $\text{AlOH}_3$ ,  
10 consisting of small unilamellar vesicles wherein the  
saponin (QS21) is in the membranes of the vesicles and  
wherein the vesicles and the antigen are absorbed onto  
a metallic salt particle  $\text{AlOH}_3$ .

All documents cited herein are hereby incorporated  
15 by reference thereto.

The immunogenic compositions typically will  
contain pharmaceutically acceptable vehicles, such as  
water, saline, glycerol, ethanol, etc. Additionally,  
auxiliary substances, such as wetting or emulsifying  
20 agents, pH buffering substances, preservatives, and  
the like, may be included in such vehicles.

Typically, the immunogenic compositions are  
prepared as injectables, either as liquid solutions or  
suspensions; solid forms suitable for solution in, or  
25 suspension in, liquid vehicles prior to injection may  
also be prepared. The preparation also may be  
emulsified or encapsulated in liposomes for enhanced  
adjuvant effect. The MSP-1<sub>42</sub> proteins of the invention  
may also be incorporated into Immune Stimulating  
30 Complexes together with saponins, for example QuilA  
(ISCOMS).

Immunogenic compositions used as vaccines  
comprise a 'sufficient amount' or 'an immunologically  
effective amount' of the proteins of the present  
35 invention, as well as any other of the above mentioned

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components, as needed. 'Immunologically effective amount', means that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment, as defined  
5 above. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated (e.g. nonhuman primate, primate, etc.), the capacity of the individual's immune system to  
10 synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, the strain of malaria infection, and other relevant factors. It is expected that the amount will fall in a  
15 relatively broad range that can be determined through routine trials. Usually, the amount will vary from 0.01 to 1000 ug/dose, more particularly from about 1.0 to 100 ug/dose most preferably from about 10 to 50 ug/dose.

20 The proteins may also serve as vaccine carriers to present homologous (e.g. other malaria antigens, such as EBA-175 or AMA-1) or heterologous (non-malaria) antigens. In this use, the proteins of the invention provide an immunogenic carrier capable of  
25 stimulating an immune response to other antigens. The antigen may be conjugated either by conventional chemical methods, or may be cloned into the gene encoding MSP-1<sub>42</sub> fused to the 5' end or the 3' end of the MSP-1<sub>42</sub> gene. The vaccine may be administered in  
30 conjunction with other immunoregulatory agents.

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby these materials, or their functional derivatives, are  
35 combined in admixture with a pharmaceutically

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acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in *Remington's Pharmaceutical Sciences* (16th ed., Osol, A. ed., Mack Easton Pa. (1980)). In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the above-described compounds together with a suitable amount of carrier vehicle.

Additional pharmaceutical methods may be employed to control the duration of action. Control release preparations may be achieved through the use of polymers to complex or absorb the compounds. The controlled delivery may be exercised by selecting appropriate macromolecules (for example polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate) and the concentration of macromolecules as well as the method of incorporation in order to control release. Another possible method to control the duration of action by controlled release preparations is to incorporate the compounds of the present invention into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating these agents into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly(methylmethacrylate)-microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and

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nanocapsules or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* (1980).

Administration of the compounds, whether  
5 antibodies or vaccines, disclosed herein may be carried out by any suitable means, including parenteral injection (such as intraperitoneal, subcutaneous, or intramuscular injection), *in ovo* injection of birds, orally, or by topical  
10 application of the antibodies (typically carried in a pharmaceutical formulation) to an airway surface. Topical application of the antibodies to an airway surface can be carried out by intranasal administration (e.g., by use of dropper, swab, or  
15 inhaler which deposits a pharmaceutical formulation intranasally). Topical application of the antibodies to an airway surface can also be carried out by inhalation administration, such as by creating respirable particles of a pharmaceutical  
20 formulation (including both solid particles and liquid particles) containing the antibodies as an aerosol suspension, and then causing the subject to inhale the respirable particles. Methods and apparatus for administering respirable particles of  
25 pharmaceutical formulations are well known, and any conventional technique can be employed. Oral administration may be in the form of an ingestible liquid or solid formulation.

The treatment may be given in a single dose  
30 schedule, or preferably a multiple dose schedule in which a primary course of treatment may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the response, for example, at 1-4  
35 months for a second dose, and if needed, a

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subsequent dose(s) after several months. Examples of suitable treatment schedules include: (i) 0, 1 month and 6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, or other  
5 schedules sufficient to elicit the desired responses expected to reduce disease symptoms, or reduce severity of disease.

The present invention also provides kits which are useful for carrying out the present invention.  
10 The present kits comprise a first container means containing the above-described antibodies. The kit also comprises other container means containing solutions necessary or convenient for carrying out the invention. The container means can be made of glass,  
15 plastic or foil and can be a vial, bottle, pouch, tube, bag, etc. The kit may also contain written information, such as procedures for carrying out the present invention or analytical information, such as the amount of reagent contained in the first container  
20 means. The container means may be in another container means, e.g. a box or a bag, along with the written information.

The present invention also relates to a method for *in vitro* diagnosis of malaria antibodies present  
25 in a biological sample, comprising at least the following steps

(i) contacting said biological sample with a composition comprising any of the MSP-1<sub>42</sub> peptides as defined above, preferably in an immobilized form under  
30 appropriate conditions which allow the formation of an immune complex, wherein said peptide or protein can be a biotinylated peptide or protein which is covalently bound to a solid substrate by means of streptavidin or avidin complexes,

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(ii) removing unbound components,  
(iii) incubating the immune complexes formed with heterologous antibodies, with said heterologous antibodies having conjugated to a detectable label

5 under appropriate conditions,

(iv) detecting the presence of said immune complexes visually or mechanically (e.g. by means of densitometry, fluorimetry, colorimetry).

The present invention also relates to a kit for  
10 determining the presence of malaria antibodies, in a biological sample, comprising:

at least one peptide or protein composition as defined above, possibly in combination with other polypeptides or peptides from *Plasmodium* or other  
15 types of malaria parasite, with said peptides or proteins being preferentially immobilized on a solid support, more preferably on different microwells of the same ELISA plate, and even more preferentially on one and the same membrane strip,

20 a buffer or components necessary for producing the buffer enabling binding reaction between these polypeptides or peptides and the antibodies against malaria present in the biological sample,

means for detecting the immune complexes formed  
25 in the preceding binding reaction,

possibly also including an automated scanning and interpretation device for inferring the malaria parasite present in the sample from the observed binding pattern.

30 The immunoassay methods according to the present invention utilize MSP-1<sub>42</sub> domains that maintain linear (in case of peptides) and conformational epitopes (proteins) recognized by antibodies in the sera from individuals infected with a malaria parasite. The MSP-  
35 1<sub>42</sub> antigens of the present invention may be employed

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in virtually any assay format that employs a known antigen to detect antibodies. A common feature of all of these assays is that the antigen is contacted with the body component suspected of containing malaria  
5 antibodies under conditions that permit the antigen to bind to any such antibody present in the component. Such conditions will typically be physiologic temperature, pH and ionic strength using an excess of antigen. The incubation of the antigen with the  
10 specimen is followed by detection of immune complexes comprised of the antigen.

Design of the immunoassays is subject to a great deal of variation, and many formats are known in the art. Protocols may, for example, use solid supports,  
15 or immunoprecipitation. Most assays involve the use of labeled antibody or polypeptide; the labels may be, for example, enzymatic, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays which amplify the signals from the immune complex are also known;  
20 examples of which are assays which utilize biotin and avidin or streptavidin, and enzyme-labeled and mediated immunoassays, such as ELISA assays.

The immunoassay may be, without limitation, in a heterogeneous or in a homogeneous format, and of a  
25 standard or competitive type. In a heterogeneous format, the polypeptide is typically bound to a solid matrix or support to facilitate separation of the sample from the polypeptide after incubation. Examples of solid supports that can be used are nitrocellulose  
30 (e.g., in membrane or microtiter well form), polyvinyl chloride (e.g., in sheets or microtiter wells), polystyrene latex (e.g., in beads or microtiter plates, polyvinylidene fluoride (known as Immunolon.TM.), diazotized paper, nylon membranes,  
35 activated beads, and Protein A beads. For example,

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Dynatech Immunolon.TM.1 or Immunlon.TM. 2 microtiter plates or 0.25 inch polystyrene beads (Precision Plastic Ball) can be used in the heterogeneous format. The solid support containing the antigenic

5 polypeptides is typically washed after separating it from the test sample, and prior to detection of bound antibodies. Both standard and competitive formats are know in the art.

10 In a homogeneous format, the test sample is incubated with the combination of antigens in solution. For example, it may be under conditions that will precipitate any antigen-antibody complexes which are formed. Both standard and competitive formats for these assays are known in the art.

15 In a standard format, the amount of malaria antibodies in the antibody-antigen complexes is directly monitored. This may be accomplished by determining whether labeled anti-xenogeneic (e.g. anti-human) antibodies which recognize an epitope on  
20 anti-malaria antibodies will bind due to complex formation. In a competitive format, the amount of malaria antibodies in the sample is deduced by monitoring the competitive effect on the binding of a known amount of labeled antibody (or other competing  
25 ligand) in the complex.

Complexes formed comprising anti-malaria antibody (or in the case of competitive assays, the amount of competing antibody) are detected by any of a number of known techniques, depending on the format. For  
30 example, unlabeled malaria antibodies in the complex may be detected using a conjugate of anti-xenogeneic Ig complexed with a label (e.g. an enzyme label).

In an immunoprecipitation or agglutination assay  
35 format the reaction between the malaria antigens and

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the antibody forms a network that precipitates from the solution or suspension and forms a visible layer or film of precipitate. If no anti-malaria antibody is present in the test specimen, no visible precipitate is formed.

There currently exist three specific types of particle agglutination (PA) assays. These assays are used for the detection of antibodies to various antigens when coated to a support. One type of this assay is the hemagglutination assay using red blood cells (RBCs) that are sensitized by passively adsorbing antigen (or antibody) to the RBC. The addition of specific antigen antibodies present in the body component, if any, causes the RBCs coated with the purified antigen to agglutinate.

To eliminate potential non-specific reactions in the hemagglutination assay, two artificial carriers may be used instead of RBC in the PA. The most common of these are latex particles. However, gelatin particles may also be used. The assays utilizing either of these carriers are based on passive agglutination of the particles coated with purified antigens.

The MSP-1<sub>42</sub> proteins, peptides, or antigens of the present invention will typically be packaged in the form of a kit for use in these immunoassays. The kit will normally contain in separate containers the MSP-1<sub>42</sub> antigen, control antibody formulations (positive and/or negative), labeled antibody when the assay format requires the same and signal generating reagents (e.g. enzyme substrate) if the label does not generate a signal directly. The MSP-1<sub>42</sub> antigen may be already bound to a solid matrix or separate with reagents for binding it to the matrix. Instructions

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(e.g. written, tape, CD-ROM, etc.) for carrying out the assay usually will be included in the kit.

Immunoassays that utilize the MSP-1<sub>42</sub> antigen are useful in screening blood for the preparation of a supply from which potentially infective malaria parasite is lacking. The method for the preparation of the blood supply comprises the following steps. Reacting a body component, preferably blood or a blood component, from the individual donating blood with MSP-1<sub>42</sub> proteins of the present invention to allow an immunological reaction between malaria antibodies, if any, and the MSP-1<sub>42</sub> antigen. Detecting whether anti-malaria antibody--MSP-1<sub>42</sub> antigen complexes are formed as a result of the reacting. Blood contributed to the blood supply is from donors that do not exhibit antibodies to the native MSP-1 antigens.

The present invention further contemplates the use of MSP-1<sub>42</sub> proteins, or parts thereof as defined above, for *in vitro* monitoring malaria infection or prognosing the response to treatment (for instance with chloroquine, mefloquine, Malarome) of patients suffering from malaria infection comprising:

incubating a biological sample from a patient with malaria infection with an MSP-1<sub>42</sub> protein or a suitable part thereof under conditions allowing the formation of an immunological complex,  
removing unbound components,  
calculating the anti-MSP-1<sub>42</sub> titers present in said sample (for example at the start of and/or during the course of therapy),  
monitoring the natural course of malaria infection, or prognosing the response to treatment of said patient on the basis of the amount anti-MSP-1<sub>42</sub> titers found in said sample at the start of treatment and/or during the course of treatment.

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Patients who show a decrease of 2, 3, 4, 5, 7, 10, 15, or preferably more than 20 times of the initial anti-MSP-1<sub>42</sub> titers could be concluded to be long-term, sustained responders to malaria therapy.

5        It is to be understood that smaller fragments of the above-mentioned peptides also fall within the scope of the present invention. Said smaller fragments can be easily prepared by chemical synthesis and can be tested for their ability to be used in an assay as  
10        detailed above.

      The present invention also relates to a kit for monitoring malaria infection or prognosing the response to treatment (for instance to medication) of patients suffering from malaria infection  
15        comprising:

          at least one MSP-1<sub>42</sub> peptide as defined above,  
          a buffer or components necessary for producing the buffer enabling the binding reaction  
          between these proteins or peptides and the anti-MSP-1<sub>42</sub>  
20        antibodies present in a biological sample,  
          means for detecting the immune complexes formed in the preceding binding reaction,  
          possibly also an automated scanning and interpretation device for inferring a decrease of  
25        anti-MSP-1<sub>42</sub> titers during the progression of treatment.

      The present invention also relates to a serotyping assay for detecting one or more serological types or alleles of malaria parasite present in a  
30        biological sample, more particularly for detecting antibodies of the different types or alleles of malaria parasites to be detected combined in one assay format, comprising at least the following steps:

          (i) contacting the biological sample to be  
35        analyzed for the presence of malaria antibodies of one

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or more serological types, with at least one of the MSP-1<sub>42</sub> compositions as defined above, preferentially in an immobilized form under appropriate conditions which allow the formation of an immune complex,

5       (ii) removing unbound components,

          (iii) incubating the immune complexes formed with heterologous antibodies, with said heterologous antibodies being conjugated to a detectable label under appropriate conditions,

10       (iv) detecting the presence of said immune complexes visually or mechanically (e.g. by means of densitometry, fluorimetry, calorimetry) and inferring the presence of one or more malaria serological types present from the observed binding pattern.

15       It is to be understood that the compositions of proteins or peptides used in this method are recombinantly expressed type-specific or allele-specific proteins or type-specific peptides.

20       The present invention further relates to a kit for serotyping one or more serological types or alleles of malaria parasite present in a biological sample, more particularly for detecting the antibodies to these serological types of malaria parasites comprising:

25       at least one MSP-1<sub>42</sub> protein or MSP-1<sub>42</sub> peptide, as defined above,

          a buffer or components necessary for producing the buffer enabling the binding reaction between these proteins or peptides and the anti-MSP-1 antibodies  
30       present in a biological sample,

          means for detecting the immune complexes formed in the preceding binding reaction,

          possibly also an automated scanning and interpretation device for detecting the presence of

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one or more serological types present from the observed binding pattern.

The present invention also relates to the use of a peptide or protein composition as defined above, for  
 5 immobilization on a solid support and incorporation into a reversed phase hybridization assay, preferably for immobilization as parallel lines onto a solid support such as a membrane strip, for determining the presence or the genotype of malaria parasite according  
 10 to a method as defined above. Combination with other type-specific or allele-specific antigens from other malaria parasites also lies within the scope of the present invention.

The contents of all cited references (including  
 15 literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

Other features of the invention will become  
 20 apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

The following MATERIALS AND METHODS were used in  
 25 the examples that follow.

Construction of expression cassette pET(AT)PfMSP-  
 1<sub>42</sub>-(3D7)

Molecular cloning and bacterial transformations were performed as described (Sambrook et al.,  
 30 Molecular cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989). The final expression construct, pETATPfMSP-142 (3D7) Tolle et al., 1995, Exp. Parasitol. 81, 47-54, was the product of a series of subclonings, each successive construction reducing

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the amount of expressed non-MSP-1 sequence. The MSP-1<sub>42</sub> fragment was prepared by PCR of genomic DNA using the forward primer GGGGATCCATTGAGGGTCGTGGTACCATGGC AATATCTGTCACAATGG (SEQ ID NO:9) and the reverse primer

5 GTCGACTTAGGAACTGCAGAAAATACCGG (SEQ ID NO:10). The product was cloned into the expression vector pMAL-p (New England Biolabs) via the 5' *Bam*HI and the 3' *Sal*I site, sequenced (SEQ ID NO:4), and then subcloned into pET32a (Novagen, Madison, Wisconsin), creating pET-

10 Trx42, which contained the MSP-1<sub>42</sub> gene fragment fused in-frame to the 3' end of the *E. coli* thioredoxin gene, *trx*. pET-Trx42 was digested with *Nde*I and religated to remove *trx*, creating pET(50)MSP-1<sub>42</sub>. pET42A was created by digesting both the pET(50)MSP-

15 1<sub>42</sub> vector and the DNA fragment GGGCATATGGCACACCATCATCATCATCCCCGGGGGATCCGAC (SEQ ID NO: 11) with *Nde*I and *Bam*HI and then ligating the two. The DNA fragment encoded six consecutive histidine residues and a short flexible linker sequence. To

20 avoid using ampicillin selection, *tet* was subcloned from pBR322 by digesting with *Eco*RI and *Pfl*MI, then blunt-ending and ligating it into the *Bst*1107 I site in pET42A creating pET-IEGR-MSP-1<sub>42</sub>(AT). The final plasmid pET(AT)PfMSP-1<sub>42</sub>(3D7) was created by removing

25 the residual Factor Xa cleavage site. This was accomplished by digesting pET-IEGR-MSP-1<sub>42</sub>(AT) and the DNA fragment GGGCATATGGCACACCATCATCATCATCCCCGGGGGAT CCGGTTCTGGTACCGAC (SEQ ID NO:12), with *Nde*I and *Kpn*I and then ligating. 3D7 MSP-1<sub>42</sub> was expressed from this

30 final construct with 17 non-MSP-1<sub>42</sub> amino acids fused to the N-terminus.

#### Expression of MSP-1<sub>42</sub>.

The expression host, BL21 DE3 (F- ompT hsdSB(rB-mB-) gal dcm (DE3)) was transformed with pETATPfMSP-

35 1<sub>42</sub>(3D7). Fresh stationary phase cultures of

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transformed bacteria were used to inoculate 1L shake  
 flasks of Super Broth containing 15 ug/ml  
 tetracycline, which were grown to 0.4 OD 600 at 37°C ,  
 cooled to temperatures ranging from 25-35°C and  
 5 induced with 0.1 mM IPTG. Cell pastes were collected  
 in lysis buffer (10 mM NaPO<sub>4</sub>, pH 6.2, 50 mM NaCl, 10  
 mM imidazole, 2 mM MgCl<sub>2</sub>, 50 U/ml benzonase) at a  
 paste to buffer ratio of 1:3 w/v. Cells were lysed by  
 microfluidization (Microfluidics) in one pass, NaCl  
 10 was added to a final concentration of 500 mM. The  
 temperature of the sample was maintained below 10°C at  
 all times. Tween-80, 1.0% w/v (final concentration),  
 was added and the lysate was centrifuged at 27,666 x g  
 for 1 hr at 4°C. Pellets and supernates were  
 15 evaluated by immunoblotting.

Bulk Fermentation and expression of MSP-1<sub>42</sub> (3D7).

A 300 L fermentor of Super Broth supplemented  
 with 15 ug/ml tetracycline was inoculated with three L  
 of fresh stationary phase culture in accordance with  
 20 Batch Production Record (BPR)-305-00. Fermentation  
 continued until an OD 600 = 4.0 - 6.0 was reached. The  
 fermentor was cooled to 25°C prior to induction with  
 0.1 mM IPTG. Three hours following induction, cells  
 were harvested by centrifugation at 15,000 rpm at 3  
 25 L/min. The cell paste was stored at -80°C in the WRAIR  
 Department of Biologics Research, Pilot Bioproduction  
 Facility.

GMP Purification of *E. coli* expressed MSP-1<sub>42</sub>  
 (3D7), BPR-335-02.

Cell paste was lysed as described  
 30 above. Tween-80 was added to 1% w/v (final), and the  
 lysate was centrifuged at 27,666 x g for 1 hr at 4°C.  
 The clarified lysate was collected and placed on ice.  
 All further steps were carried out at 4°C.

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Ni<sup>+2</sup> NTA Superflow (Qiagen, Germany): A column  
 with a 6:1 w/v cell paste to resin ratio, was  
 equilibrated with 10 mM NaPO<sub>4</sub>, pH 6.2, 500 mM NaCl, 10  
 mM imidazole (Ni-buffer) supplemented with 0.5% Tween  
 80 (w/v). The clarified lysate was applied at a flow  
 rate of 30 ml/min and the column washed with 1.1  
 volumes of Ni-buffer containing 0.5% Tween-80. The  
 column was then washed with 30 volumes of Ni-buffer  
 containing 0.5% Tween 80 (w/v); 20 volumes of 10 mM  
 NaPO<sub>4</sub>, pH 6.2, 75 mM NaCl, 10 mM imidazole; and 15  
 volumes of 10 mM NaPO<sub>4</sub>, pH 8.0, 75 mM NaCl, 20 mM  
 imidazole. MSP-1<sub>42</sub> was eluted with 10 volumes 10 mM  
 NaPO<sub>4</sub>, pH 8.0, 75 mM NaCl, 160 mM imidazole, and was  
 diluted with an equal volume of 10 mM NaPO<sub>4</sub>, 75 mM  
 NaCl, pH 8.0, and 0.4% Tween 80.

Q ion exchange chromatography: A Toyopearl  
 SuperQ 650 M (TosoHaas) column (cell paste: resin  
 ratio = 3:1 w/v), was equilibrated with 10 mM NaPO<sub>4</sub>,  
 pH 8.0, 75 mM NaCl, 80 mM imidazole, 0.2% Tween 80 (Q-  
 buffer). The diluted sample was applied at a flow  
 rate of 30 ml/min and washed with one volume of Q-  
 buffer, which was combined with the flow through to  
 pool the MSP-1<sub>42</sub>, giving a final volume of this pool  
 equal to 50 Q column volumes. This sample was diluted  
 with an equal volume of 10 mM NaPO<sub>4</sub>, pH 6.0, 0.4%  
 Tween 80 (v/v), and the pH of the sample was adjusted  
 to pH 6.0 with 6N HCl.

CM ion exchange chromatography: A CM 650 M  
 (TosoHaas) column (cell paste:resin ratio = 2:1) was  
 equilibrated with 10 mM NaPO<sub>4</sub>, pH 6.0, 35 mM NaCl,  
 0.2% Tween 80 (CM-equilibration buffer) and the sample  
 was applied at a flow rate of 30 ml/min. The column  
 was washed with 6 volumes of CM-equilibration buffer,  
 followed by 10 volumes of 10mM NaPO<sub>4</sub>, pH 7.0, 100 mM  
 NaCl, 0.02% Tween 80 (v/v). The MSP-1<sub>42</sub> was eluted

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with 10 mM NaPO<sub>4</sub>, pH 7.2, 250 mM NaCl in three column volumes.

SDS-PAGE and Immunoblotting. Protein samples were separated under reducing (10% 2-mercaptoethanol) or nonreducing conditions by SDS-PAGE with Tris-Glycine buffering (Invitrogen). Protein was detected by Coomassie Blue R250 staining. Immunoblotting was performed with nitrocellulose membranes (Invitrogen) blocked using 5% nonfat dry milk and 0.1% Tween 20 in PBS, pH 7.4. Blots were probed with polyclonal rabbit anti-MSP-1<sub>42</sub> antibodies or mAbs diluted into phosphate buffered saline, pH 7.4 containing 0.1% Tween 20. This buffer was also used for washing. The second antibodies were alkaline phosphatase-conjugated anti-rabbit IgG or anti-mouse IgG (H+L) (Promega, Madison, Wisconsin) and reactions were detected with nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemicals, St. Louis, Missouri) in 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 100 mM Tris-HCl, pH 9.5. mAbs used for evaluation of structure included 2.2 (Mackay et al., 1985, Embo J. 4, 3823-3829), 12.8 (Conway et al, 1991, Parasitology 103, 1-6), 7.5 (McBride et al., 1982, Science 217, 254-257), 12.10 (Blackman et al., 1990, supra), 5.2 (Chang et al., 1988, Exp. Parasitol. 67, 1-11), and 7F1 (Lyon et al., 1987, J. Immunol. 138, 895-901).

#### Antigen Stability Studies

Stability studies were conducted for 18 months on MSP-1<sub>42</sub> stored at 4°C, -20°C, and -80°C and for lyophilized product stored at -20°C. Stability was evaluated by Commassie Blue staining and immunoblotting of SDS PAGE gels run under non-reduced and reduced conditions.

#### Vaccine Preparation

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The CM eluate was concentrated two-fold to 0.5 mg/ml, the buffer was exchanged with 10 volumes of phosphate buffered saline by diafiltration and the protein was sterilized by filtration through a Millipak 60 0.22-um filtration unit. Final bulk antigen (FMP1) was stored at -80°C. To prepare the antigen for use with adjuvant ADJUVANT B (GlaxoSmithKline Biologicals, Rixensart, Belgium), 118.5 ml of sterile purified FMP1 was mixed with 236 ml of 50 mM Na Phosphate, 101 ml of 15.75% lactose, and 0.5 ml Tween-80, producing formulated antigen at a concentration of 118.5 mg/ml. Formulated FMP1 was sterile filtered with Millipak 40 0.22-um filtration unit and added to 3 ml vials for lyophilization. The vials were sealed with Lyo stoppers and metal crimps (BPR-334-01, Lot 0678)

#### Vaccine Potency

Female Balb/C mice were immunized subcutaneously with 100 ul of vaccine. Potency studies were performed with 1.0, 0.3 and 0.1 ug of MSP-1<sub>42</sub> in Adjuvant B (GlaxoSmithKline Biologicals, Rixensart, Belgium). The 10 ug dose was prepared with 10 ug of antigen and 100 ul of Adjuvant B and was used to make the 1.0 and 0.1 ug doses by diluting into 0.9% saline. Mice were primed and then bled and boosted four- weeks later, and bled again 2 weeks following boosting. Sera were analyzed by ELISA and results are reported in ELISA units, or the serum dilution that gives an absorbance of 1 OD 405 (Stoute et al., 1997, N. Engl. J. Med. 336, 86-91).

Seroconversion occurred if the following condition was met: (ELISA Units-3SD)post-vaccination - (ELISA Units+3SD)pre-vaccination > 0

#### Safety and Immunogenicity

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Rhesus monkeys were vaccinated intramuscularly with 50 ug of antigen formulated with ADJUVANT B or alum. Monkeys were boosted one, three, five, and seven months after priming and sera were collected prior to and two weeks following each immunization.

#### Serology

Sera were analyzed by IFA against methanol fixed 3D7 strain *P. falciparum* schizont-infected erythrocytes (Lyon et al., 1987, supra) and by kinetic ELISA. For this, the MSP-1<sub>42</sub> capture antigen was diluted in PBS at pH 7.4 and coated at 0.4 pmoles /well overnight at 4°C, and wells were blocked with CaseinBlock (Pierce). Sera were diluted 1:50,000 (ADJUVANT B) or 1:8,000 (Alum) in CaseinBlock and reacted for 1 h at room temperature, followed by reaction with alkaline phosphatase-conjugated rabbit anti-human IgG (H&L) (Promega) diluted 1:250 in CaseinBlock also for 1 hr at room temperature. Detection of p-nitrophenyl phosphate substrate conversion to product was measured at 5 min intervals for 30 min. The slope of the line was calculated by linear regression and R<sup>2</sup> was at least 0.99 for each analysis.

#### Inhibition of Parasite Invasion

Rabbits were vaccinated four times subcutaneously with FMP1 in Freund's adjuvants and sera were collected three weeks after the final immunization. The IgG fractions of pre-immune and post-immune sera were prepared with Protein-G chromatography and quantified by using the Bradford Protein Determination assay (Pierce). For the invasion inhibition assay (Chulay et al., 1981, Am. J. Trop. Med. Hyg. 30, 12-19), the IgG fractions were dialyzed against RPMI 1640

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adjusted to pH 7.4 with NaOH and added to settled 100  
 ul cultures of synchronous *P. falciparum* (3D7 strain)  
 schizont-infected erythrocytes (3-5 nuclei) at 2%  
 hematocrit and 0.25% parasitemia. In some  
 5 experiments, native FMP1 or reduced and alkylated  
 FMP1, dialyzed as above, were added as specificity  
 controls to reverse the activity of inhibitory  
 antibodies.

### Example 1

10 The gene fragment containing *P. falciparum* 3D7  
 strain MSP-1<sub>42</sub> was cloned into pET32a (Figure 1A) with  
 several modifications. These included adding the gene  
 for tetracycline, removing the *trx* gene and other  
 elements, and adding a hexa-histidine affinity tag,  
 15 which contained 17 non-MSP-1<sub>42</sub> amino acids fused to the  
 N-terminus of MSP-1<sub>42</sub> (Figure 1B). Our primary  
 objective of expressing MSP-1<sub>42</sub> as a soluble protein in  
*E. coli* cytoplasm was achieved by systematically  
 varying IPTG concentration and induction temperature.  
 20 IPTG at 0.1 mM induced maximal protein synthesis and  
 induction at 25°C was required to express soluble  
 protein (data not shown). The optimal biomass density  
 for induction in 10L fermentors was shown to be 4-6  
 OD600. These conditions were used to prepare GMP cell  
 25 paste in a 300 L fermentor, and this paste was used to  
 develop the purification process. The MSP-1<sub>42</sub> was  
 purified by three chromatographic steps. After  
 centrifugation, cleared lysates were applied to a Ni<sup>+2</sup>-  
 NTA Superflow resin for affinity purification (Figure  
 30 2A, lane 1). This step removed most of the endotoxin,  
 which depended on extensive washing of bound protein  
 at low pH (pH 6.2) and high sodium chloride (500 mM).  
 After this step, the protein was greater than 50% pure  
 by densitometry (arrows show MSP-1<sub>42</sub> related bands).  
 35 MSP-1<sub>42</sub> was purified to greater than 95% purity by

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chromatography on a Q-anion exchanger followed by a  
CM-cation exchanger (Figure 2A, lanes 2 and 3  
respectively). The Final Bulk Antigen (FMP1) was  
further characterized by SDS-PAGE under non-reducing  
5 and reducing conditions (Figure 2B, lanes 1 and 2,  
respectively) and by immunoblotting with rabbit anti-  
*E. coli* antibody (Figure 2C, lane 3) and MSP-1  
specific mAbs. The MSP-1<sub>33</sub> specific mAb 7F1 reacted  
with two proteins migrating at 36kD and 38kD (Figure  
10 2C, lane 1), but the MSP-1<sub>19</sub> specific mAb 12.10 (Figure  
2C, lane 2) did not, nor did any of the other MSP-1<sub>19</sub>  
specific mAbs. Full-length MSP-1<sub>42</sub> (see Figure 2) and  
all of the higher molecular weight aggregates were  
reactive against all the MSP-1 specific mAbs used.  
15 Long term stability studies showed that the FMP1 was  
stable when stored for 18 months at -80°C but not when  
stored at 4°C (degradation) or -20°C (aggregation)  
(Figure 3, lanes 1-3, respectively), compare with  
Figure 2B (lane 1).

## 20 **Example 2**

Vaccine potency studies were conducted in Balb/C  
mice immunized with FMP1 formulated for use with  
adjuvant ADJUVANT B. At the 0.3 ug and 1.0 ug doses,  
respectively, all mice seroconverted following one  
25 immunization. At the 0.1 ug dose 50% of the mice  
seroconverted following the first immunization; all  
seroconverted after the second (data not shown).  
Safety and immunogenicity of the product was assessed  
in Rhesus monkeys immunized up to five times with 50  
30 ug doses of FMP1/ADJUVANT B (n=8) or FMP1/alum (n=6).  
No adverse local responses were observed and all  
biochemical and hematological laboratory tests were  
normal for both groups (not shown). FMP1/ADJUVANT B  
induced malaria parasite reactive IFA titers that  
35 increased to 1:28,000 by two weeks following the

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second immunization and maintained this level through the third immunization (Figure 4, right ordinate). The fourth immunization induced a brief increase in titer, which returned to the 1:28,000 base within six weeks. The fifth immunization induced a response that was similar to the fourth. FMP1/alum induced IFA titer that paralleled those induced by FMP1/ADJUVANT B but were six-fold lower. FMP1/ADJUVANT B induced MSP-1<sub>42</sub>-specific ELISA-reactive antibodies that increased to 2100 OD/min by two weeks following the second immunization and gradually increase to a maximum of 2800 OD/min after the fourth immunization (Figure 4, left ordinate). The geometric mean antibody level after five immunizations was lower than after four, but this difference was not significant. By four weeks after each immunization antibody levels fell about 30% but stabilized after the third immunization. FMP1/alum induced ELISA-reactive antibodies levels roughly paralleled those induced by FMP1/ADJUVANT B but were about ten-fold lower. FMP1 in Freund's adjuvants induced invasion inhibitory IgG antibodies. The inhibition was titratable (Figure 5) and was completely reversed by adding competing soluble FMP1 at a final concentration of 17 ug/ml and partially reversed by adding reduced and alkylated FMP1 (approximately 50% reversal) at the same concentration (data not shown).

### Example 3

FMP1 combined with the adjuvants ADJUVANT A, ADJUVANT B ADJUVANT C, ADJUVANT E, Adjuvant D, and Alum were assessed for safety and immunogenicity in Rhesus monkeys at the human dose of 50 ug/injection.

Studies were conducted in adult *Macaca mulatta* housed at the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. Monkeys

were screened to exclude animals in poor health or with previous exposure to malaria, and were monitored for at least 6 weeks prior to the start of the study. Animals were randomly assigned to treatment groups, and vaccines were administered in a blinded standardized fashion while animals were under ketamine anesthesia.

Animals received intramuscular injections of FMP1 (50 ug) combined with adjuvant at 0, 4, 12 weeks. The attending veterinarian who conducted clinical and laboratory evaluations assessed vaccine safety. Blood was obtained for CBC and serum chemistries prior to administration and at 24, 48, and 72 hours immediately after each immunization. Local reactogenicity was monitored at the time of immunization, day 1, day 2, day 3, and day 14 post-immunization.

#### **Safety**

The following safety data apply to FMP1 combined with ADJUVANT B. The same data are available for the other adjuvants as well.

Clinical evaluations before and after each immunization revealed minimal local and no systemic toxicities. Reactogenicity of FMP1 in ADJUVANT B was assessed in each Rhesus monkey using the following criteria: 1) skin warmth or calor; 2) skin erythema or rubor; 3) skin swelling or edema; 4) muscle induration; 5) muscle necrosis. The intensity of the reactions were scored as follows: 0 = none; 1 = slight or mild reaction; 2 = moderate reaction; 3 = marked or severe reaction. The mean prevalence (Table 2A) and mean intensity (Table 2B) of local reactogenicity is given in the tables below. Erythema, skin swelling, and muscle induration were limited to the site of inoculation and resolved in all monkeys by 14 days post immunization. There were no cases of muscle

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necrosis. The intensity of muscle induration diminished daily from post immunization day 1 through day 3, and multiple immunizations did not increase the risk of adverse reactions. Behavior, activity, and food consumption remained normal.

Table 2A

| Mean Prevalence of Reactogenicity- mean % positive (n = 8 Rhesus) |           |                       |                    |                      |                      |                   |                 |
|---|-----------|-----------------------|--------------------|----------------------|----------------------|-------------------|-----------------|
| Immunization Number   |           | Post-immunization day | Skin warmth/ calor | Skin erythema/ rubor | Skin swelling/ edema | Muscle induration | Muscle necrosis |
| 1   | 10-Aug-99 | -                     | 0%                 | 0%                   | 0%                   | 0%                | 0%              |
|   | 11-Aug-99 | 1                     | 50%                | 12.5%                | 37.5%                | 87.5%             | 0%              |
|   | 12-Aug-99 | 2                     | 0%                 | 0%                   | 12.5%                | 75%               | 0%              |
|   | 13-Aug-99 | 3                     | 0%                 | 0%                   | 0%                   | 12.5%             | 0%              |
|   | 24-Aug-99 | 14                    | 0%                 | 0%                   | 0%                   | 0%                | 0%              |
| 2   | 7-Sep-99  | -                     | 0%                 | 0 of 8               | 0 of 8               | 0 of 8            | 0%              |
|   | 8-Sep-99  | 1                     | 12.5%              | 87.5%                | 100%                 | 100%              | 0%              |
|   | 9-Sep-99  | 2                     | 0%                 | 50%                  | 100%                 | 100%              | 0%              |
|   | 10-Sep-99 | 3                     | 0%                 | 12.5%                | 87.5%                | 100%              | 0%              |
|   | 21-Sep-99 | 14                    | 0%                 | 0%                   | 0%                   | 0%                | 0%              |
| 3   | 9-Nov-99  | -                     | 0 of 8             | 12.5%                | 2 of 8               | 0 of 8            | 0%              |
|   | 10-Nov-99 | 1                     | 37.5%              | 25%                  | 75%                  | 100%              | 0%              |
|   | 11-Nov-99 | 2                     | 12.5%              | 0%                   | 37.5%                | 100%              | 0%              |
|   | 12-Nov-99 | 3                     | 0%                 | 0%                   | 0%                   | 75%               | 0%              |
|   | 23-Nov-99 | 14                    | 0%                 | 0%                   | 0%                   | 0%                | 0%              |

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Table 2B

| Immunization Number |           | Post-immunization day | Skin warmth/ calor | Skin erythema/ rubor | Skin swelling/ edema | Muscle induration | Muscle necrosis |
|---------------------|-----------|-----------------------|--------------------|----------------------|----------------------|-------------------|-----------------|
| 1                   | 10-Aug-99 | -                     | 0                  | 0                    | 0                    | 0                 | 0               |
|                     | 11-Aug-99 | 1                     | 1                  | 1                    | 1.3                  | 1.3               | 0               |
|                     | 12-Aug-99 | 2                     | 0                  | 0                    | 1                    | 1                 | 0               |
|                     | 13-Aug-99 | 3                     | 0                  | 0                    | 0                    | 1                 | 0               |
|                     | 24-Aug-99 | 14                    | 0                  | 0                    | 0                    | 0                 | 0               |
| 2                   | 7-Sep-99  | -                     | 0                  | 0                    | 0                    | 0                 | 0               |
|                     | 8-Sep-99  | 1                     | 2                  | 1.6                  | 3                    | 2.9               | 0               |

|   |           |    |   |     |     |     |   |
|---|-----------|----|---|-----|-----|-----|---|
|   | 9-Sep-99  | 2  | 0 | 1.8 | 3   | 2.4 | 0 |
|   | 10-Sep-99 | 3  | 0 | 1   | 2.6 | 2.3 | 0 |
|   | 21-Sep-99 | 14 | 0 | 0   | 0   | 0   | 0 |
|   |           |    |   |     |     |     |   |
| 3 | 9-Nov-99  | -  | 0 | 0   | 0   | 0   | 0 |
|   | 10-Nov-99 | 1  | 1 | 1.5 | 1.3 | 2.1 | 0 |
|   | 11-Nov-99 | 2  | 1 | 0   | 1   | 1.6 | 0 |
|   | 12-Nov-99 | 3  | 0 | 0   | 0   | 1.5 | 0 |
|   | 23-Nov-99 | 14 | 0 | 0   | 0   | 0   | 0 |

Mean laboratory values for the entire group (Tables 3 and data not shown) indicated no significant abnormalities or trends in hematologic or biochemical laboratory tests. Quantitative platelet counts varied before and after immunization. This variation occurred both within individual Rhesus monkeys and between Rhesus. All blood prior to December 1999 was collected in heparin anti-coagulant, and we suspected that the platelet count variation was attributed to the use of the heparin anticoagulant. All Rhesus in the study had blood samples collected in mid December 1999 using both heparin and EDTA anticoagulants and quantitative platelet counts determined at 2 locations in Bangkok Thailand. AFRIMS veterinary pathologists read all blood smears from August, September, and November and the platelet counts were read as adequate, increased, or decreased. It was decided that all further hematologic (CBC) laboratory assays would be collected in EDTA. The January 2000 samples reflect this change in blood collection procedure with normal platelet counts observed in all Rhesus immunized with FMP1 and ADJUVANT B days 1, 2, and 3 following a 4<sup>th</sup> immunization. In addition, all blood smears at each time point reviewed after immunizations 1, 2, and 3 had adequate platelet counts (data not shown).

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### Immunogenicity

**Humoral Immunity:** FMP1 combined with ADJUVANT A or ADJUVANT B induced similar levels of ELISA (Table 4A) and IFA (Table 4B) reactive antibody and were significantly more potent than the other adjuvant combinations. FMP1 combined with Alum was the least immunogenic by ELISA after two immunizations, but was not different from ADJUVANT C, ADJUVANT E and Adjuvant D by ELISA after the third immunization. FMP1 combined with Alum or with ADJUVANT C induced similar levels of IFA reactive antibody and induced significantly more IFA reactive antibody than FMP1 combined with ADJUVANT E or Adjuvant D.

**Cellular Immunity:** FMP1 combined with ADJUVANT A or ADJUVANT B induced different cytokine response profiles (Table 5). Both vaccines induced comparable levels of IFN- $\gamma$  response after the 3<sup>rd</sup> dose, however, the IL-5 response was greatly suppressed in animals receiving FMP1 combined with ADJUVANT A. Duration of T cell response data indicate that IFN- $\gamma$  response generated by FMP1 combined with both ADJUVANT A and ADJUVANT B persists at least 24 weeks after vaccination. The difference on Th1/Th2 polarization of FMP1 formulated with ADJUVANT B and ADJUVANT A was not anticipated.

Table 3: Mean Biochemical Laboratory Test Values

|                    | Immunization #1 |      |      |      | Immunization #2 |      |      |      | Immunization #3 |      |      |      |
|--------------------|-----------------|------|------|------|-----------------|------|------|------|-----------------|------|------|------|
|                    | August          |      |      |      | September       |      |      |      | November        |      |      |      |
|                    | 5               | 11   | 12   | 13   | 7               | 8    | 9    | 10   | 9               | 10   | 11   | 12   |
| BUN (mg/ml)        |                 |      |      |      |                 |      |      |      |                 |      |      |      |
| MEAN               | 19.7            | 17.3 | 18.1 | 19.0 | 20.0            | 16.9 | 20.6 | 17.9 | 17.9            | 18.5 | 16.3 | 16.4 |
| s.d.               | 3.5             | 3.4  | 2.4  | 3.7  | 2.9             | 2.6  | 2.2  | 2.9  | 2.9             | 1.1  | 2.4  | 1.9  |
| CREATININE (mg/ml) |                 |      |      |      |                 |      |      |      |                 |      |      |      |
| MEAN               | 0.76            | 0.81 | 0.80 | 0.81 | 0.80            | 0.83 | 0.75 | 0.81 | 0.88            | 0.90 | 0.83 | 0.86 |
| s.d.               | 0.07            | 0.04 | 0.11 | 0.06 | 0.09            | 0.12 | 0.08 | 0.11 | 0.18            | 0.09 | 0.10 | 0.12 |
| AST (U/l)          |                 |      |      |      |                 |      |      |      |                 |      |      |      |
| MEAN               | 23.9            | 80.8 | 67.9 | 61.9 | 27.4            | 47.0 | 50.1 | 47.6 | 27.8            | 68.8 | 57.3 | 46.0 |
| s.d.               | 3.7             | 38.9 | 22.5 | 24.4 | 4.1             | 13.6 | 20.3 | 17.1 | 4.5             | 23.3 | 18.5 | 14.9 |
| ALT (U/l)          |                 |      |      |      |                 |      |      |      |                 |      |      |      |
| MEAN               | 28.0            | 50.9 | 50.1 | 56.3 | 32.8            | 37.3 | 39.5 | 43.9 | 38.4            | 41.5 | 47.0 | 51.5 |
| s.d.               | 9.1             | 16.0 | 15.2 | 17.1 | 13.3            | 13.6 | 12.7 | 12.9 | 10.6            | 13.7 | 12.8 | 13.5 |
| CPK (U/l)          |                 |      |      |      |                 |      |      |      |                 |      |      |      |
| MEAN               | 301             | 1192 | 879  | 923  | 256             | 791  | 671  | 470  | 307             | 2443 | 915  | 520  |
| s.d.               | 239             | 550  | 684  | 657  | 208             | 356  | 298  | 237  | 368             | 1039 | 721  | 221  |

Table 4A : Rhesus Humoral Responses to FMP1 formulated with various adjuvants

|    |            | Week 0*              | Week 2       | Week 4*      | Week 6               | Week 13*      | Week 15        |
|----|------------|----------------------|--------------|--------------|----------------------|---------------|----------------|
| 5  |            |                      |              |              | Midpoint ELISA Titer |               |                |
|    | ADJUVANT A | GMT 13<br>95% CI 17  | 1402<br>1413 | 1824<br>1171 | 52161<br>15783       | 9291<br>4088  | 74348<br>20937 |
| 10 | ADJUVANT B | GMT 7<br>95% CI 2    | 2439<br>657  | 3929<br>2164 | 87096<br>24748       | 25574<br>6797 | 98458<br>42826 |
| 15 | ADJUVANT C | GMT 27<br>95% CI 101 | 325<br>597   | 715<br>714   | 31654<br>15885       | 2576<br>633   | 25120<br>9183  |
|    | ADJUVANT E | GMT 6<br>95% CI 3    | 80<br>155    | 186<br>234   | 25360<br>9517        | 2698<br>915   | 35945<br>10550 |
| 20 | ADJUVANT D | GMT 5<br>95% CI 24   | 245<br>315   | 392<br>259   | 16899<br>16810       | 1799<br>2113  | 23647<br>12386 |
|    | Alum       | GMT 8<br>95% CI 21   | 64<br>129    | 98<br>118    | 5501<br>7168         | 751<br>865    | 10969<br>11011 |

25 \*Immunization time point

Table 4B

|    | Week 0*    | Week 2 | Week 4* | Week 6 | Week 13* | Week 15 |
|----|------------|--------|---------|--------|----------|---------|
| 5  |            |        |         |        |          |         |
|    | GMT 0      | 4000   | 1308    | 26251  | 11888    | 43069   |
|    | 95% CI 0   | 2079   | 7492    | 5410   | 6372     | 11853   |
| 10 |            |        |         |        |          |         |
|    | GMT 0      | 3668   | 4362    | 26909  | 19027    | 26909   |
|    | 95% CI 0   | 1283   | 1793    | 5132   | 6584     | 5132    |
| 15 |            |        |         |        |          |         |
|    | GMT 0      | 24     | 917     | 9514   | 2594     | 10375   |
|    | 95% CI 488 | 257    | 849     | 6458   | 0        | 6187    |
|    | GMT 14     | 12     | 15      | 144    | 31       | 257     |
|    | 95% CI 3   | 4      | 6       | 98     | 29       | 131     |
| 20 |            |        |         |        |          |         |
|    | GMT 50     | 54     | 74      | 675    | 169      | 844     |
|    | 95% CI 13  | 19     | 21      | 319    | 66       | 238     |
|    | GMT 0      | 316    | 794     | 2828   | 1414     | 4490    |
|    | 95% CI 0   | 470    | 0       | 4158   | 839      | 3719    |

25 \*Immunization time point

Table 5 : Polarization by ADJUVANT A of Immune Responses to FMP1

| <u>Adjuvant</u> | <u>IFN-<math>\gamma</math>/IL-5</u> |
|-----------------|-------------------------------------|
| ADJUVANT A      | 4.2                                 |
| ADJUVANT B      | 0.75                                |
| ADJUVANT C      | 2.3                                 |
| ADJUVANT E      | 0.77                                |
| Adjuvant D      | 5.0                                 |
| Alum            | 0.53                                |

#### 5 **Example 4**

A Phase I dose escalation clinical trial of the recombinant Plasmodium falciparum malaria vaccine candidate FMP1/ADJUVANT B was recently completed at the Walter Reed Army Institute of Research (WRAIR) in Silver Spring, MD in 15 adult human volunteers to assess safety, reactogenicity, and immunogenicity. This vaccine was created by researchers at the WRAIR and manufactured at the Pilot Bioproduction Facility at WRAIR. In the initial Phase I clinical trial conducted during 4th quarter 2000 - 1st quarter 2001, three groups of 5 volunteers were immunized with 1/5th dose, 1/2 dose, or full dose of vaccine at 0, 1, and 3 months. Tables 1 and 2 below summarize the demographics of the study population.

20

#### **Safety and Adverse Events:**

Local and general systemic adverse events were assessed at 6 time points following each immunization, and blood tests to evaluate hematologic, renal, and hepatic abnormalities were performed before and after each immunization. The vaccine proved safe in all 15 volunteers with NO serious adverse events or clinical laboratory abnormalities noted. There were no drop-outs from the vaccine trial. All adverse events were graded according to severity. There were no Serious

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Adverse Events (SAE's) requiring hospitalization nor were there any grade 3 adverse events as defined below. Grade "3" = Adverse experience which prevents normal everyday activities and necessitates a  
 5 corrective therapy. The specific occurrences by subject and by dose of all adverse events are summarized in tables 3-7 below. In summary, the most frequent adverse events was minimal pain at the site of vaccine injection which disappeared by 24 hours post-  
 10 inoculation.

#### **Immunogenicity:**

##### **ELISA:**

The vaccine was extremely potent in inducing high-titer antibody responses in all volunteers as  
 15 assessed by ELISA (enzyme-linked immunoabsorbent assay). The table below summarizes the mean, standard deviation, and geometric mean antibody titers for each of the three vaccine groups.

20 Table 6- ANTIBODY TITERS to MSP-1<sub>42</sub> BY ELISA

VALUES INDICATE DILUTION OF SERA WHICH GIVES OD = 1

|    |           |       |        |        |        |        |        |
|----|-----------|-------|--------|--------|--------|--------|--------|
|    | 1/5 Dose  | Day 0 | Day 14 | Day 28 | Day 42 | Day 84 | Day 98 |
| 25 | Average   | 12    | 312    | 462    | 18066  | 7371   | 32648  |
|    | Std Dev   | 8     | 191    | 236    | 8406   | 6304   | 24046  |
|    | Geo Mean  | 10    | 272    | 412    | 16440  | 5749   | 26626  |
|    | 1/2 DOSE  | Day 0 | Day 14 | Day 28 | Day 42 | Day 84 | Day 98 |
| 30 | Average   | 28    | 1285   | 2530   | 44172  | ND     | 57771  |
|    | Std Dev   | 6     | 1882   | 2631   | 21176  | ND     | 24192  |
|    | Geo Mean  | 28    | 636    | 1762   | 40744  | ND     | 53569  |
|    | Full Dose | Day 0 | Day 14 | Day 28 | Day 42 | Day 84 | Day 98 |
| 35 | Average   | 32    | 688    | 990    | 32461  | 15914  | 50053  |
|    | Std Dev   | 32    | 414    | 306    | 19307  | 5650   | 29991  |
|    | Geo Mean  | 22    | 586    | 951    | 28448  | 14938  | 42799  |

**IFA:** The vaccine was also immunogenic as assessed by indirect immunofluorescence titers to malaria parasites.

5

Table 7- IMMUNOFLUORESCENCE to 3D7 Parasites  
Values indicate serum dilution which gives 1+ IFA intensity to methanol-fixed malaria parasites

10

## DAY OF STUDY

|    |                       |       |        |        |        |        |        |
|----|-----------------------|-------|--------|--------|--------|--------|--------|
|    | 1/5 Dose-<br>GROUP 1  | Day 0 | Day 14 | Day 28 | Day 42 | Day 84 | Day 98 |
|    | Geomean               | 200   | 200    | 200    | 1393   | 2111   | 3676   |
| 15 | 95% CI                |       |        |        | 1152   | 2533   | 5155   |
|    | SD                    | 0     | 0      | 0      | 1315   | 2890   | 5881   |
|    | 1/2 Dose-<br>GROUP 2  | Day 0 | Day 14 | Day 28 | Day 42 | Day 84 | Day 98 |
|    | Geomean               | 200   | 200    | 200    | 2425   | ND     | 5572   |
| 20 | 95% CI                |       |        |        | 768    | ND     | 1254   |
|    | SD                    | 0     | 0      | 0      | 876    | ND     | 1431   |
|    | Full Dose-<br>GROUP 3 | Day 0 | Day 14 | Day 28 | Day 42 | Day 84 | Day 98 |
|    | Geomean               | 200   | 200    | 200    | 3200   | 3676   | 8445   |
| 25 | 95% CI                |       |        |        | 1536   | 1882   | 3967   |
|    | SD                    | 0     | 0      | 0      | 1753   | 2147   | 4525   |

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In addition, cell-mediated immunologic responses were noted in the majority of vaccinated subjects. Peripheral blood mononuclear cells were stimulated with MSP-1 antigen or *P. falciparum* parasitized erythrocytes and proliferation was measured by uptake of <sup>3</sup>H-thymidine. Figure 6 shows the results of PBMC proliferation in each of the subjects after each dose of vaccine.

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**Summary**

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In this initial clinical trial involving a small number of volunteers, FMP1/ADJUVANT B has been shown to be a safe and highly immunogenic vaccine that elicits both parasite-reactive antibodies and cellular

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responses. This study develops a foundation to further test its safety profile and evaluate its efficacy to reduce morbidity and mortality in target populations directly affect by *P. falciparum* malaria.

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### SAFETY DATA

Table 8: Number of subjects

|  | TOTAL | Percent | Group1 | Group 2 | Group 3 |
|--|-------|---------|--------|---------|---------|
| Number of subjects planned                 | 15    | 100%    | 5      | 5       | 5       |
| Subjects or vaccine number not allocated   | 0     | 0       | 0      | 0       | 0       |
| Number of subjects enrolled (Total cohort) | 15    | (100%)  | 5      | 5       | 5       |
| "Reasons for elimination..."               |       |         |        |         |         |
| Group 1: FMP1/ADJUVANT B: 1/5th dose       |       |         |        |         |         |
| Group 2: FMP1/ADJUVANT B: 1/2 dose         |       |         |        |         |         |
| Group 3: FMP1/ADJUVANT B: full dose        |       |         |        |         |         |

Table 9: Demographics : Study population

| Sex    | N  | Mean Age (years) | Min age (years) | Max. Age (years) | S.D. (years) |
|--------|----|------------------|-----------------|------------------|--------------|
| Female | 5  | 32.2             | 27              | 51               | 10.8         |
| Male   | 10 | 34.4             | 22              | 52               | 11.1         |
| Total  | 15 | 33.7             | 22              | 52               | 10.7         |

N = total number of subjects

S.D. = standard deviation

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Table 10- Incidence and nature of symptoms reported per dose and per subject after vaccination

| Dose           | Group | N | General symptoms |    | Local symptoms |    |
|----------------|-------|---|------------------|----|----------------|----|
|                |       |   | n                | %  | n              | %  |
| <i>By dose</i> |       |   |                  |    |                |    |
| Dose 1         | 15    |   | 2                | 13 | 12             | 80 |
| Dose 2         | 15    |   | 2                | 13 | 11             | 73 |
| Dose 3         | 15    |   | 1                | 7  | 10             | 67 |
| Overall        | 45    |   | 5                | 11 | 33             | 73 |

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Table 10 Continued

| Table 10 Continued |  |   |                  |    |                |     |
|--------------------|--|---|------------------|----|----------------|-----|
| Dose               | Group  | N | General symptoms |    | Local symptoms |     |
|                    |  |   | n                | %  | n              | %   |
| 5                  | <i>By volunteer</i>  |   |                  |    |                |     |
|                    | Group 1  | 5 | 1                | 20 | 4              | 80  |
|                    | Group 2  | 5 | 1                | 20 | 4              | 80  |
|                    | Group 3  | 5 | 2                | 40 | 5              | 100 |
| 10                 | N per dose = Number of documented doses                              |   |                  |    |                |     |
|                    | N per volunteer = Number of volunteers                               |   |                  |    |                |     |
|                    | n per dose = Number of documented doses with at least one symptom    |   |                  |    |                |     |
|                    | n per subject = Number of subjects with at least one documented dose |   |                  |    |                |     |

Table 11- Incidence of solicited local symptoms including symptoms graded at maximum intensity

| Solicited local Symptom | Intensity    | Group 1<br>(N=15) |      | Group 2<br>(N=15) |      | Group 3<br>(N=15) |      |
|-------------------------|--------------|-------------------|------|-------------------|------|-------------------|------|
|                         |              | n                 | %    | n                 | %    | n                 | %    |
| Pain                    | Total        | 8                 | 53.3 | 11                | 73.3 | 14                | 93.3 |
|                         | grade "3"    | 0                 | 0    | 0                 | 0    | 0                 | 0    |
| Redness                 | Total        | 1                 | 6.7  | 4                 | 26.7 | 2                 | 13.3 |
|                         | > 50 mm/>24h | 0                 | 0    | 0                 | 0    | 0                 | 0    |
| Swelling                | Total        | 0                 | 0    | 0                 | 0    | 0                 | 0    |
|                         | > 50 mm/>24h | 0                 | 0    | 0                 | 0    | 0                 | 0    |

Group 1: FMP1/ADJUVANT B: 1/5th dose

Group 2: FMP1/ADJUVANT B: 1/2 dose

Group 3: FMP1/ADJUVANT B: full dose

N = Total number of documented doses

n = number of documented doses after which there is at least one report of a symptom

% = percentage of documented doses after which there is at least one report of a symptom

Grade "3" = pain preventing normal daily activity and necessitates a corrective therapy

&gt;50mm/&gt;24h = redness or swelling with a diameter of more than 50 mm and persisting for more than 24 hours

Table 12: Subjects reporting solicited local symptoms including symptoms graded at maximum intensity

| 5  | Solicited local symptom  | Intensity    | Group 1<br>(N=5) |    | Group 2<br>(N=5) |    | Group 3<br>(N=5) |     |
|----|--|--------------|------------------|----|------------------|----|------------------|-----|
|    |  |              | n                | %  | n                | %  | n                | %   |
|    | Pain   | Total        | 4                | 80 | 4                | 80 | 5                | 100 |
|    |  | grade "3"    | 0                | 0  | 0                | 0  | 0                | 0   |
| 10 | Redness  | Total        | 1                | 20 | 3                | 60 | 2                | 40  |
|    |  | > 50 mm/>24h | 0                | 0  | 0                | 0  | 0                | 0   |
|    | Swelling   | Total        | 0                | 0  | 0                | 0  | 0                | 0   |
|    |  | > 50 mm/>24h | 0                | 0  | 0                | 0  | 0                | 0   |
| 15 | Group 1: FMP1/ADJUVANT B: 1/5th dose<br>Group 2: FMP1/ADJUVANT B: 1/2 dose<br>Group 3: FMP1/ADJUVANT B: full dose<br>N = Total number of subjects<br>n = number of subjects reporting at least one report of a symptom<br>%= percentage of subjects reporting at least symptom<br>Grade "3" = pain preventing normal daily activity and necessitates a corrective therapy<br>>50mm/>24h = redness or swelling with a diameter of more than 50 mm and persisting for more than 24 hours |              |                  |    |                  |    |                  |     |
| 20 |  |              |                  |    |                  |    |                  |     |
| 25 |  |              |                  |    |                  |    |                  |     |

Table 13: Incidence of solicited general symptoms including symptoms graded at maximum intensity and those probably or suspected of being related to vaccination

| 30 | Symptoms   |                   | Group 1<br>N=15 |   | Group 2<br>N=15 |   | Group 2<br>N=15 |     |
|----|------------|-------------------|-----------------|---|-----------------|---|-----------------|-----|
|    |            |                   | N               | % | N               | % | N               | %   |
| 35 | Arthralgia | Total             | 0               | 0 | 0               | 0 | 1               | 6.7 |
|    |            | PB/SU             | 0               | 0 | 0               | 0 | 0               | 0   |
|    |            | PB/SU & Grade "3" | 0               | 0 | 0               | 0 | 0               | 0   |
| 40 | Fever      | Total             | 0               | 0 | 0               | 0 | 1               | 6.7 |
|    |            | PB/SU             | 0               | 0 | 0               | 0 | 0               | 0   |
|    |            | PB/SU & Grade "3" | 0               | 0 | 0               | 0 | 0               | 0   |
| 45 | Headache   | Total             | 0               | 0 | 0               | 0 | 0               | 0   |
|    |            | PB/SU             | 0               | 0 | 0               | 0 | 0               | 0   |
|    |            | PB/SU & Grade "3" | 0               | 0 | 0               | 0 | 0               | 0   |

Table 13 Continued

| 5  | Symptoms  |                      | Group 1<br>N=15 |     | Group 2<br>N=15 |     | Group 2<br>N=15 |     |
|----|-----------|----------------------|-----------------|-----|-----------------|-----|-----------------|-----|
|    |           |                      | N               | %   | N               | %   | N               | %   |
| 10 | Malaise   | Total                | 1               | 6.7 | 1               | 6.7 | 1               | 6.7 |
|    |           | PB/SU                | 1               | 6.7 | 1               | 6.7 | 0               | 0   |
|    |           | PB/SU &<br>Grade "3" | 0               | 0   | 0               | 0   | 0               | 0   |
| 15 | Myalgia   | Total                | 1               | 6.7 | 0               | 0   | 1               | 6.7 |
|    |           | PB/SU                | 1               | 6.7 | 0               | 0   | 0               | 0   |
|    |           | PB/SU &<br>Grade "3" | 0               | 0   | 0               | 0   | 0               | 0   |
| 20 | Rash      | Total                | 0               | 0   | 0               | 0   | 0               | 0   |
|    |           | PB/SU                | 0               | 0   | 0               | 0   | 0               | 0   |
|    |           | PB/SU &<br>Grade "3" | 0               | 0   | 0               | 0   | 0               | 0   |
| 25 | Dizziness | Total                | 0               | 0   | 0               | 0   | 1               | 6.7 |
|    |           | PB/SU                | 0               | 0   | 0               | 0   | 0               | 0   |
|    |           | PB/SU &<br>Grade "3" | 0               | 0   | 0               | 0   | 0               | 0   |
| 30 | Nausea    | Total                | 0               | 0   | 0               | 0   | 1               | 6.7 |
|    |           | PB/SU                | 0               | 0   | 0               | 0   | 0               | 0   |
|    |           | PB/SU &<br>Grade "3" | 0               | 0   | 0               | 0   | 0               | 0   |

Group 1: FMP1/ADJUVANT B: 1/5th dose

Group 2: FMP1/ADJUVANT B: \* dose

Group 3: FMP1/ADJUVANT B: full dose

35 N = Total number of documented doses.

n= number of documented doses after which there is at least one report of a symptom.

%= percentage of documented doses after which there is at least one report of a symptom.

40 Grade "3" = Adverse experience which prevents normal everyday activities and necessitates a corrective therapy.

PB /SU= Probably or suspected of an association. -

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Table 14: Subjects reporting solicited general symptoms including symptoms graded at maximum intensity and those probably or suspected of being related to vaccination

|    |            |                   | Group 1<br>(1/5 dose)<br>N=5 |    | Group 2<br>(1/2 dose)<br>N=5 |    | (Full dose)<br>Group 2<br>N=5 |    |
|----|------------|-------------------|------------------------------|----|------------------------------|----|-------------------------------|----|
|    |            |                   | N                            | %  | N                            | %  | N                             | %  |
| 10 | Symptoms   |                   |                              |    |                              |    |                               |    |
|    | Arthralgia | Total             | 0                            | 0  | 0                            | 0  | 1                             | 20 |
|    |            | PB/SU             | 0                            | 0  | 0                            | 0  | 0                             | 0  |
|    |            | PB/SU & Grade "3" | 0                            | 0  | 0                            | 0  | 0                             | 0  |
| 15 | Fever      | Total             | 0                            | 0  | 0                            | 0  | 1                             | 20 |
|    |            | PB/SU             | 0                            | 0  | 0                            | 0  | 0                             | 0  |
|    |            | PB/SU & Grade "3" | 0                            | 0  | 0                            | 0  | 0                             | 0  |
| 20 | Headache   | Total             | 0                            | 0  | 0                            | 0  | 0                             | 0  |
|    |            | PB/SU             | 0                            | 0  | 0                            | 0  | 0                             | 0  |
|    |            | PB/SU & Grade "3" | 0                            | 0  | 0                            | 0  | 0                             | 0  |
| 25 | Fatigue    | Total             | 1                            | 20 | 1                            | 20 | 1                             | 20 |
|    |            | PB/SU             | 1                            | 20 | 1                            | 20 | 0                             | 0  |
|    |            | PB/SU & Grade "3" | 0                            | 0  | 0                            | 0  | 0                             | 0  |
| 30 | Myalgia    | Total             | 1                            | 20 | 0                            | 0  | 1                             | 20 |
|    |            | PB/SU 1           | 20                           | 0  | 0                            | 0  | 0                             | 0  |
|    |            | PB/SU & Grade "3" | 0                            | 0  | 0                            | 0  | 0                             | 0  |
| 35 | Rash       | Total             | 0                            | 0  | 0                            | 0  | 0                             | 0  |
|    |            | PB/SU             | 0                            | 0  | 0                            | 0  | 0                             | 0  |
|    |            | PB/SU & Grade "3" | 0                            | 0  | 0                            | 0  | 0                             | 0  |
| 40 | Dizziness  | Total             | 0                            | 0  | 0                            | 0  | 1                             | 20 |
|    |            | PB/SU             | 0                            | 0  | 0                            | 0  | 0                             | 0  |
|    |            | PB/SU & Grade "3" | 0                            | 0  | 0                            | 0  | 0                             | 0  |
| 45 | Nausea     | Total             | 0                            | 0  | 0                            | 0  | 1                             | 20 |
|    |            | PB/SU             | 0                            | 0  | 0                            | 0  | 0                             | 0  |
|    |            | PB/SU & Grade "3" | 0                            | 0  | 0                            | 0  | 0                             | 0  |

N = Total number of subjects.

n= number of subject reporting at least one report of a symptom.

%= percentage of subject reporting at least one report of a symptom.

Grade "3" = Adverse experience which prevents normal everyday activities and necessitates a corrective therapy.

PB /SU= Probably or suspected of an association.

## Example 5

Three rabbits per immunization group were vaccinated 4 times at 3-week intervals with 50 µg FMP1 (3D7) in Montanide adjuvant, subcutaneously, or with FMP1 in ADJUVANT B, intramuscularly. Two negative control rabbits per group were immunized with each adjuvant alone. A final control rabbit was immunized with reduced and alkylated MSP1<sub>42</sub> (3D7) in Montanide. Each rabbit was bled from the ear vein 2 weeks following each immunization. Following the fourth immunization the rabbits were ex-sanguinated from the heart and the sera from these rabbits was analyzed by MSP1 (3D7)-specific antigen ELISA's.

The sera were analyzed by MSP1 (3D7)-specific ELISA and by kinetic ELISA. The MSP1-specific capture antigen was diluted in PBS at pH 7.4 and coated at 0.4pmoles/well overnight at 4°C and the wells were blocked with CaseinBlock (Pierce). Sera were first diluted by 1:25 and then followed by two-fold serial dilutions down the plate up to  $1.6 \times 10^6$  fold. Sera were reacted for 1 hour at room temperature, followed by reaction with alkaline phosphatase-conjugated goat anti-rabbit IgG (H&L) (Promega) diluted 1:5,000 in CaseinBlock for 1 hour at room temperature. Detection of p-nitrophenyl phosphate substrate conversion to product was measured at 60 minutes. The data are reported as the average of triplicate values plotted from the titration curve measured at OD<sub>405</sub>.

Immunization with FMP1 in Montanide and FMP1 in ADJUVANT B induces high MSP1<sub>42</sub> specific antibody titers following the second immunization. The geometric mean of the post fourth immunization MSP1<sub>42</sub> specific antibody titers induced by immunization with FMP1/ADJUVANT B and FMP1/Montanide were 1: 363,000 and

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1:182,000, respectively. Neither adjuvant/antigen combination substantially boosted the MSP1<sub>42</sub> specific antibody titers after a third and fourth immunization.

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#### DISCUSSION

Here we describe the development, fermentation, expression, purification and characterization of a safe and immunogenic recombinant MSP-1<sub>42</sub> that is suitable for production of antibodies and for use in diagnostic assays and as a potential vaccine.

MSP-1<sub>42</sub> which was derived from recombinant *E. coli* was highly purified and met all FDA standards necessary for testing safety and immunogenicity in humans. Endotoxin levels were significantly below the FDA acceptable levels (FDA; 350 EU/dose/70kg human, Our Process; 9.14 EU/dose/70kg human). Residual levels of all chemicals used in the purification process were quantified and determined to be within levels set as production specifications. Although the MSP-1<sub>42</sub> comprised greater than 95% of the protein in the final product, it was not homogeneous due to proteolysis at the C-terminal end of the protein. This proteolysis appeared to occur during expression in the *E. coli* host because long term studies showed that the antigen was stable when stored at -80°C for 18 months, revealing little change in the Coomassie Blue staining pattern or mAb reaction patterns in immunoblots (Figure 3).

Previous studies have shown that the induction of antibody responses to epitopes on MSP-1<sub>19</sub> correlate with clinical immunity to malaria (Egan et al., 1996, J. Infect. Dis. 173, 765-769), suggesting that induction of such responses depends on correctly forming the disulfide-dependent conformational

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epitopes present within the MSP-1<sub>19</sub> portion of MSP-1<sub>42</sub>. This conclusion is further supported by the observation that the only MSP-1<sub>19</sub> specific mAbs capable of inhibition merozoite invasion react with

5 conformational epitopes (Burghaus et al., 1994, Mol. Biochem. Parasitol. 64, 165-169). Recombinant MSP-1<sub>42</sub> has correct structure because it reacted with all of the MSP-1<sub>19</sub> specific mAbs we used; these were raised against malaria parasites and included functional mAbs

10 classified as growth or invasion inhibitory (mAb 12.10, 12.8) (Blackman et al., 1990, supra) and blocking inhibitory mAbs (mAb 7.5, 2.2, 1E1) (Guevara et al., 1997, J. Exp. Med. 186, 1689-1699).

These data support the continued use of bacterial

15 systems for expressing soluble malaria antigens that contain conformational epitopes as potential vaccine candidates.

In addition, FMP1/ADJUVANT B was highly immunogenic in the Rhesus monkeys as well as Balb/C

20 mice. After three immunizations the geometric mean IFA antibody titers induced in Rhesus monkeys exceeded 1:36,000 and the geometric mean ELISA titer exceed 26,000 OD/min (Figure 4); these titers did not change with further immunization. FMP1/ADJUVANT B induced

25 approximately six times more MSP-1 specific antibodies than FMP1/Alum (Figure 4). MSP-1 specific antibodies induced by FMP1 were predominantly against MSP-1<sub>42</sub> and MSP-1<sub>19</sub> compared to MSP-1<sub>33</sub> (not shown). This result, when taken in combination with FMP1's ability to

30 induce high titer antibodies that react with schizont-infected erythrocytes and it's ability to induce invasion inhibitory antibodies (Figure 5 and Table 1) further indicates that it has correct structure. Currently, the only malaria vaccine that has

35 reproducibly protected human volunteers is the

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RTS,S/ADJUVANT B vaccine (Kester et al., 2001, J. Infect. Dis. 183, 640-647). When combined with ADJUVANT B, FMP1 was highly immunogenic and caused no adverse biochemical or local reactions in mice.

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